THE NEONATAL COLOSTRUM-DEPRIVED PIGLET: AN ANIMAL MODEL FOR INVESTIGATING NEONATAL NUTRITION AND CARNITINE METABOLISM

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1988

ACKNOWLEDGEMENTS

I wish to thank the following persons who have helped to make this project possible:

The members of my doctoral committee, Dr. Fuller W. Bazer, Dr. Richard P. Boyce, Dr. Rose D. Brown, Jr., Dr. Jesse F. Gregory, III, and Dr. John A. Koburger, for their helpful suggestions and critiques of this work.

My advisor, Dr. Peggy R. Borum, for her patience, support, and encouragement throughout the course of this study.

My husband, James David Sapp, III, for his patience, understanding, and support throughout the duration of my graduate studies.

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Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

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April, 1988

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The animal models available to study neonatal nutrition are inadequate. Research concerning the critical importance of certain nutrients to neonatal well-being is therefore limited. Carnitine is required for the β-oxidation of long chain fatty acids and is involved in the initiation of ketogenesis. Carnitine may function in the regulation of gluconeogenesis, branched chain amino acid catabolism and ammonia metabolism.

A better understanding of neonatal carnitine metabolism could be gained with the development of an animal model more closely resembling the human neonate. The piglet is similar to the infant in physiology, relative maturity at birth and susceptibility to hypothermia and hypoglycemia. This study sought to establish the neonatal colostrum-deprived piglet as a model for neonatal carnitine metabolism. Specific aims of this project were to investigate the gestational patterns of carnitine accretion in the piglet and to ascertain the viability of the colostrum-deprived piglet by developing technique for their maintenance in a neonatal intensive care unit environment. In addition, the tissue uptake patterns of tracer carnitine given by intravenous and enteral routes were investigated in this neonatal model.

Results obtained support the use of this model to study neonatal carnitine metabolism. These include the establishment of similar carnitine accretion patterns in the tissues of both species during gestation. Like the human neonate, the total carnitine levels in the piglet skeletal muscle increase with increasing gestational age, while plasma and erythrocyte levels decrease. Methods for hand-feeding, surgical catheterization, and maintenance of viable colostrum-deprived piglets were developed. Profiles of tissue uptake of radioactivity from tracer carnitine administered intravenously and enterally were investigated. These profiles suggest that similar patterns of carnitine uptake occur in certain tissues. Radioactivity accumulates slowly in heart and skeletal muscle and more rapidly in the liver and kidney. These patterns may reflect the particular metabolism or function of carnitine in the tissues.

CHAPTER 1

INTRODUCTION

Evidence suggests that carnitine is a nutrient of critical importance to the human neonate. This is due to the established and proposed roles of carnitine in many aspects of neonatal metabolism. Carnitine is required for the transport of long chain fatty acids into the inner mitochondria to the site of β-oxidation (1). Fat becomes a major metabolic fuel source for the neonate soon after birth. Carnitine is also involved in other metabolic processes of importance to the neonate such as ketogenesis and thermogenesis (1). In addition, evidence suggests that carnitine is involved in other processes which are important to the neonate such as gluconeogenesis (1) and ammonia metabolism (2,3). Carnitine may have a role in lung maturation in late fetal or early neonatal life (4,5). During gestation, carnitine accumulates in the skeletal muscle of the human fetus (6). Both colostrum and milk contain substantial levels of carnitine (7). For this reason, many non-milk based infant formulas in this country are now being supplemented with carnitine. Human infants may have a limited ability to synthesize carnitine (8). This suspected developmental aspect of carnitine synthesis probably poses no metabolic threat to the healthy human infant receiving breast milk or carnitine supplemented infant formula. Certain infants, however, may be compromised in terms of carnitine status. Premature infants may be delivered before an adequate carnitine level in the skeletal muscle (presumably resulting from maternal transfer) has accumulated. Premature or sick neonates usually do not receive breast milk. Often oral feeds cannot be tolerated by the infant and nutritional support is maintained parenterally. At present, total parenteral nutrition solutions in this country are not carnitine supplemented. Premature infants appear to have a limited ability to utilize fat as a metabolic fuel. The production of ketones, another potential metabolic fuel, is also apparently limited in these infants. Poor gluconeogenic control and hypoglycemia is also a problem in premature or sick infants. Likewise, problems in maintaining normal ammonia levels (hyperammonemia) and in thermoregulation are often observed. For the above reasons, carnitine supplementation to premature or sick infants is under consideration. Information regarding neonatal carnitine metabolism is needed to determine the optimal methods of carnitine supplementation to such patients.

The potential routes of carnitine supplementation to the premature or sick infant include the intravenous and the intragastric (enteral) routes. For optimal supplementation it would be helpful to have an understanding of neonatal metabolism or compartmentation of carnitine administered by these two routes. Information about neonatal carnitine metabolism is needed but realistically cannot be gained through direct experimentation on human infants. A full understanding of the infant's compartmentation and metabolism of carnitine would require extensive tissue sampling. Samples would be needed from the many potential tissue pools of carnitine such as liver, kidney, cardiac and skeletal muscle, gastrointestinal tract, bile and other tissues. In addition, the use of radioactively labeled carnitine to trace the tissue uptake of carnitine would provide valuable information on carnitine movement in the neonatal body. Clearly, such extensive sampling and the use of radiolabeled tracer would be impossible in human infants.

The development of an experimental animal model with which to further investigate the metabolism of carnitine and other nutrients by the neonate would therefore be of value. The neonatal colostrum-deprived piglet represents a viable candidate for such a model. Piglets have been proposed as models for use in pediatric studies (9). Advantages of piglets relative to rodent models include body size and similarities to humans in physiology (9). In addition, both piglets and human neonates share similarities in metabolism and metabolic weaknesses such as susceptibility to hypoglycemia, hypothermia, infection and dehydration (10).

It is well known that both species rely heavily on fat as an energy source soon after birth (10). Colostrum and milk of the pig contain considerable carnitine (11). Ideally, there should be evidence of similar carnitine metabolism during fetal life between the species. A finding of similar patterns of carnitine accretion during gestation would suggest that both human infants and neonatal piglets begin extrauterine life in a similar carnitine status.

The optimal neonatal piglet model for these studies would be deprived of maternal colostrum and milk ingestion. Deprivation of colostrum but feeding of a carnitine-free diet would help to create an animal model in a carnitine status similar to the premature or sick human infant residing in a neonatal intensive care unit. This neonatal colostrum-deprived piglet model would necessarily have to survive both colostrum-deprivation and whatever surgical procedures would be required to allow tracer carnitine administration and blood and urine collection.

With these goals in mind, the specific aims of these investigations will be threefold. The first specific aim will be to determine the carnitine accretion patterns throughout gestation in the fetal piglet in order to compare the patterns to those observed in the human fetus. The second specific aim will be to maintain and use colostrum-deprived neonatal piglets in a neonatal intensive care environment by developing methods and procedures for their delivery, feeding, surgical catheterization and radiolabeled tracer carnitine administration. The final specific aim of this study will be to utilize the neonatal colostrum-deprived piglet and the newly developed methods to investigate the patterns of tissue accumulation of radioactivity from radiolabeled tracer carnitine administered intravenously or intragastrically in the neonate.

CHAPTER 2

REVIEW OF LITERATURE

Established and Proposed Functions of Carnitine

Introduction

Carnitine is a quaternary amine, β-hydroxy-γ-trimethylaminobutyric acid (12). Carnitine is synthesized from trimethyllysine in human liver, kidney, and brain tissue (13) Established functions for carnitine include its role in the transport of long chain fatty acids into the mitochondrial matrix (for subsequent β-oxidation) and its involvement in brown adipose tissue thermogenesis (1). Carnitine may be involved in the regulation of gluconeogenesis (1). In addition, carnitine functions in the initiation of ketogenesis (1) and may be involved in branched chain amino acid metabolism (1). Other proposed functions for carnitine include a role as an acetyl-CoA:CoA buffer (13), a treatment for certain inborn errors of metabolism (14) and ammonia toxicity (2,3), and a role as an appetite stimulant (1).

Carnitine and β-Oxidation

Carnitine is required for the transport of long chain fatty acids into the mitochondria to the site of β-oxidation.

The stimulatory effect of carnitine on β-oxidation was established by the ability of carnitine to increase fatty acid oxidation in perfused liver and in skeletal and heart muscle homogenate fractions (13). Acetylcarnitine and fatty acid esters of carnitine were found to be good substrates of the enzymes of rat kidney, testis, brain, heart, and liver mitochondrial β-oxidation (15).

A further understanding of this stimulatory effect was gained with the identification of carnitine acyltransferase (16). The enzymes involved in the carnitine-dependent transfer of fatty acids into the mitochondria include an outer and inner carnitine palmitoyltransferase (17) and the carnitine acylcarnitine translocase enzyme (18). Carnitine palmitoyltransferase I (Outer) catalyzes the transfer of activated fatty acyl groups from acyl-CoA to carnitine, thus forming acylcarnitine. The carnitine acylcarnitine translocase enzyme then transfers this acylcarnitine across the inner mitochondrial membrane in exchange for free carnitine (18). Carnitine acyltransferase II (Inner) then catalyzes the formation of inner mitochondrial fatty acyl-CoA which is accessible to the enzymes of β-oxidation.

While the carnitine acyltransferase enzyme does exhibit a wide range of substrate specificity (C-2 to C-22) (19), it is generally believed that the mitochondrial acyltransferase is a palmitoyltransferase (13). Carnitine acyltransferase has been found associated with peroxisomes; these enzyme activities appear to be carnitine acetyltransferase and carnitine octanyltransferase activities (20). It has been reported that β-oxidation of long chain fatty acids does occur in the peroxisomes (21). Several laboratories have suggested that carnitine may thus function to shuttle short and medium chain acyl residues out of the peroxisomes (20,21).

Carnitine in Brown Fat Thermogenesis

Carnitine is apparently of great importance in the metabolism of brown adipose tissue (BAT). Subcutaneously injected (^{14}C)-D,L-carnitine or (^{3}H)-L-carnitine accumulated in the BAT of late fetal or young suckling rats more rapidly than in heart or muscle (22). Cold-acclimated (5°C) rats have a body pool of carnitine that is eight times greater than that of rats maintained at 25°C (23). The oxidation of endogenous substrate (fatty acids) by brown fat mitochondria is carnitine dependent (24). The observation of a lack of coupling between mitochondrial respiration (electron transport) and phosphorylation (24) in BAT mitochondria has led to an understanding of the importance of BAT in heat production (non-shivering thermogenesis)

Carnitine and Gluconeogenesis

Carnitine may have a role in the regulation of gluconeogenesis (1). Patients with genetic carnitine deficiency have severe episodes of hypoglycemia (25). The compound responsible for "Jamaican Vomiting Sickness," hypoglycin (L-α-amino-β-methylenecyclopropanepropionic acid) causes hypoglycemic convulsions and coma when ingested (26). Hypoglycin has been found to depress carnitine palmitoyltransferase activity (26). Carnitine addition to myocardial homogenates from hypoglycin treated mice restored the fatty acid oxidation rates and the carnitine palmitoyltransferase activity to normal (26). Administration of carnitine to hypoglycin treated mice prevented the decrease in palmitate oxidation, carnitine palmitoyltransferase activity, and the hypoglycemia (26). Carnitine derivatives of fatty acids have been shown to stimulate net glucose production in a variety of tissue preparations, including pigeon liver homogenates, rat liver slices, and perfused rat liver (27). The stimulatory effect of oleic acid on glucose production from lactate in perfused livers from fasted rats was completely blocked by (+)decanoylcarnitine, a known inhibitor of carnitine palmitoyltransferase (28). Octanoate oxidation and stimulation of gluconeogenesis was not blocked by (+)decanoylcarnitine. The authors also observed a block in the normal changes in gluconeogenic and citric acid cycle intermediates induced by oleic acid in the presence of (+)decanoylcarnitine. These observations led the authors to conclude that the stimulatory effect of fatty acids on gluconeogenesis is determined by the rate of fatty acid oxidation (28). Similarly, (-)-fatty acylcarnitines, (-)-decanoylcarnitine, and (-)-palmitoylcarnitine were found to stimulate ^{14}C incorporation into glucose from 2-(^{14}C)-lactate and U-(^{14}C)-alanine in pigeon liver homogenates (29). These authors proposed that the carnitine acyltransferase (palmitoyltransferase) system should be considered as a possible regulatory site for gluconeogenesis. Perhaps the acetyl-CoA derived from carnitine dependent β-oxidation stimulated the pyruvate carboxylase reaction (or existed in a mitochondrial acetyl-CoA pool more readily available to the pyruvate carboxylase (29). Another proposed action was the inhibition of pyruvate dehydrogenase by the acetyl-CoA produced by the carnitine stimulated β-oxidation. In this situation, the conversion of pyruvate to acetyl-CoA would be inhibited, this would result in the stimulation of the pyruvate carboxylase reaction (29). More recently, Ferré and coworkers concluded that in newborn rats hepatic fatty acid oxidation can increase the rate of gluconeogenesis by providing acetyl-CoA necessary for the pyruvate carboxylase reaction and by providing the reducing equivalents necessary to displace the glyceraldehyde 3-phosphate dehydrogenase reaction in the direction of gluconeogenesis (30).

Carnitine and Ketogenesis

Carnitine is involved in the initiation of ketogenesis. The addition of carnitine to liver homogenates from fasting guinea pigs resulted in a twofold increase in acetoacetate production (31). McGarry (12) noted that treatments which cause enhancement of ketogenic capacity such as anti-insulin serum, glucagon, starvation, or alloxan-induced diabetes also result in an elevation of the liver carnitine concentration. The stimulation of ketogenesis cannot be due to increased carnitine concentration alone, however. Perfused livers of lactating, fed rat dams showed only a small increase in ketogenesis from oleate relative to virgin rat controls despite elevations in liver carnitine concentrations (33). It was proposed that hepatic glycogen levels were also an important factor. Depletion of glycogen reserves (fasting) also stimulated ketogenesis (34). Maximal stimulation of ketogenesis might occur with increased carnitine levels coupled to decreased glycogen reserves (34,35). McGarry suggests that the stimulatory effect of carnitine on ketogenesis occurs via increased activity of carnitine acyltransferase (I). This enzyme has been found to be elevated in liver preparations from ketotic rats (34). The "hormonal imbalance" or increased glucagon:insulin ratio in ketotic states may result in either increased hepatic synthesis of carnitine or increased carnitine mobilization to the liver (35) and may stimulate the carnitine acyltransferase enzyme (34,35). This enzyme stimulation may result from suppression of malonyl-CoA production and lipogenesis (35). Malonyl-CoA is a potent inhibitor of carnitine acyltransferase. Thus suppression of malonyl-CoA production and lipogenesis results in the activation of fatty acid oxidation and ketogenesis. Increased hepatic carnitine concentration and increased fatty acyl-CoA levels enhance β-oxidation and the production of acetoacetate and 3-hydroxybutyric acid (34,35).

Carnitine in Branched Chain Amino Acid Metabolism

Carnitine appears to have a stimulatory effect on the decarboxylation of branched chain amino acids and/or on the subsequent oxidation of their branched chain oxo-acids (36,37). L-carnitine increased the oxidation of branched chain 2-oxo-acids derived from leucine and valine in a concentration-dependent manner in intact mitochondria of rat skeletal muscle (37). Considerable amounts of branched chain acylcarnitines also accumulated in the media (37). Similarly, Choi and coworkers (38) found increased levels of isobutyrylcarnitine and isovalerylcarnitine in the skeletal muscle of a-day fasted adult male rats. Addition of carnitine or 3-methylbutyrylcarnitine had little affect on the branched chain 2-oxo-acid oxidation in mitochondria of liver, brain, or kidney cortex but had a marked effect on the heart mitochondria (37). It was proposed that the intramitochondrial ratio of acylcarnitine and acyl-CoA ester affects the activity of branched chain 2-oxo-acid dehydrogenase (37). May et al. (39) concluded that the branched chain oxoacid dehydrogenase exists on the inner mitochondrial membrane. This enzyme catalyzes the production of CO_2 and acyl-CoA esters. The acyl-CoA ester thus produced is inhibitory on the branched chain dehydrogenase. Carnitine then combines with the acyl-CoA to form an acylcarnitine ester which is transported out of the inner mitochondrial space (39). Carnitine stimulated the oxidation of 2-oxoisocaproate in intact rat skeletal muscle mitochondria; the accumulation of isovalerylcarnitine in the media led these workers to propose that carnitine stimulated the removal of these isovaleryl residues from the mitochondria for oxidation elsewhere (40). Paul and Adibi (36) found that carnitine stimulated the α-decarboxylation of leucine and valine in gastrocnemius muscle of fed rats. These workers concluded that carnitine stimulated the decarboxylation of branched chain amino acids and the conversion of their ketoanalogs to carnitine esters by one of two mechanisms: first, the carnitine stimulated transport of isovaleryl-CoA into the mitochondria shifts the equilibrium of the dehydrogenase reaction to the right (in the direction of increased branched chain amino acid decarboxylation). The second proposed mechanism was that carnitine, by freeing CoA (in the production of carnitine esters), increased the availability of free CoA for the branched chain amino acid dehydrogenase reaction. Finally, Bremer (13) suggests that the formation of branched chain acylcarnitines is a "dead-end" reaction. He concludes that the α-keto acid dehydrogenase must be located inside the CoA permeation barrier because mitochondria can decarboxylate branched chain α-ketoacids in the absence of carnitine and external CoA and NAD. Thus, any carnitine esters would have to be transferred back in the mitochondria for further metabolism. Bremer further suggests that the mitochondrial formation of branched chain acylcarnitines may be another part of the buffering effect of carnitine on mitochondrial acyl-CoA/CoA ratios.

Carnitine as an Acetyl-CoA:CoA Buffer

Bremer has proposed that carnitine may have a role as a buffer for excess acetyl units (13). Carnitine may help relieve "acetyl pressure" or alter the ratio of free CoA:acetyl-CoA (13,41). In starved sheep, the liver ratio of free CoA:acetyl-CoA was 0.34:1 (41). The levels of acetyl-CoA in the starved sheep were the same as in the normal sheep, but the acetylcarnitine levels were five times greater. These workers proposed that carnitine acetyltransferase allows the "acetyl pressure" to shift from the CoA system to the carnitine system. Similar proposals have been made for perfused rat heart (42). Thus carnitine may provide a buffer or "sink" for mitochondrial acetyl units. This has been proposed for sheep liver, rat heart, frog muscle, and blowfly flight muscle (13). The carnitine concentration in these tissues is very much greater than the

CoA concentration, thus the extramitochondrial acetylcarnitine:carnitine ratio prevents fluctuations in the mitochondrial acetyl-CoA/CoA ratio. Additionally, there may be some stimulation of acetyl-CoA oxidation by carnitine in mitochondria of rat heart and pig kidney (13). Thus, it has been suggested that carnitine may be involved in the oxidation of excess extramitochondrial acetyl-CoA, perhaps acetyl-CoA of peroxisomal origin (13).

Carnitine and Inborn Errors of Metabolism

The role of carnitine in the treatment of certain inborn errors of metabolism appears similar to its role in the relief of "acetyl pressure." It has been proposed that there may be beneficial effects of carnitine supplementation to patients with organic acidurias (14). In methylmalonic aciduria and propionic acidemia there is an accumulation of propionyl-CoA (14). With carnitine supplementation to these patients there is an increased urinary excretion of propionylcarnitine and acetylcarnitine (14). This may allow for the release of CoA which otherwise would be sequestered with the accumulation of acyl-CoA metabolites, resulting in a perturbation of metabolic homeostasis. Patients with these metabolic disorders have three primary problems which may be alleviated with carnitine supplementation (14). First, these patients have a lack of CoA availability due to the sequestering in acyl-CoA compounds. Second, they have decreased ATP production. Third, they appear to have insufficient L-carnitine levels to meet their increased requirements. Similarly, in isovaleric acidemia there are increased levels of isovaleryl-CoA and insufficient L-carnitine levels. Carnitine supplementation resulted in increased urinary levels of isovalerylcarnitine (43). The clinical improvement in response to L-carnitine challenges was thought to provide evidence for the beneficial effects of carnitine in restoring free CoA levels and intramitochondrial homeostasis (43).

Carnitine and Ammonia Metabolism

Carnitine may be involved in ammonia metabolism. L-carnitine injected into mice 30 minutes prior to an LD_{100} dose of ammonium acetate reduced mortality by 100% and prevented the appearance of symptoms of ammonia toxicity (2). There were decreased brain ammonia levels and partial restoration of brain glutamate levels with L-carnitine pretreatment. In mice with sustained hyperammonia induced by urease injections, the blood ammonia concentrations of those treated with L-carnitine were two times higher than levels in mice dying of acute ammonia intoxication (3). The carnitine treated mice, however, displayed no symptoms of ammonia toxicity. One possible explanation of these effects of carnitine may be that the carnitine stimulated intramitochondrial generation of reducing equivalents overcomes an ammonia-induced block in the malate-aspartate shuttle. This would lead to increased ATP production, thus overcoming one of the proposed causes of ammonia toxicity (3). Also, carnitine may promote urea synthesis by stimulating the production of acetyl-CoA. Increased acetyl-CoA could in turn result in increased synthesis of N-acetylglutamate and thereby activate carbamyl phosphate synthetase. In this way urea synthesis and ammonia utilization would be increased (3).

Carnitine may be involved in other aspects of brain metabolism. A transport system for carnitine has been identified in rat brain (44) This system is competitively inhibited by γ-aminobutyric acid (GABA) (44). Thus, carnitine may help to regulate the relative neuronal concentration of GABA. It has been proposed that acetylcarnitine transports the acetyl groups required for acetylcholine into the cytosol. Carnitine may therefore be required for acetylcholine synthesis (44).

Carnitine and Appetite Stimulation

Carnitine appears to have an as yet unexplained stimulatory effect on appetite. Carnitine supplementation of children has been associated with increased growth rates and appetite (45,46). Fraenkel et al. (47) cites several instances of a stimulatory effect of carnitine on the exocrine pancreas and biliary secretions of experimental animals. The question of whether there is a relationship between these carnitine-stimulated phenomena remains to be answered.

Carnitine and Neonatal Metabolism

Introduction

Carnitine plays a critical role in several different aspects of neonatal metabolism. In the newborn mammal, carnitine is important in energy metabolism through its involvement in fatty acid oxidation, ketogenesis, and lipolysis. Carnitine is also required for non-shivering thermogenesis in brown or "brown-type" adipocytes. Besides these roles, carnitine may also be involved in neonatal nitrogen metabolism by exerting an effect on ureogenesis and ammonia homeostasis. The developmental patterns of carnitine and the enzymes of carnitine biosynthesis and metabolism in late gestation and early extrauterine life indicate both the importance of and critical need for carnitine by the neonate.

Neonatal Carnitine Status

There is evidence that maternal to fetal transfer of carnitine occurs during gestation. Pregnant women have lower plasma carnitine levels than nonpregnant women (48). Free carnitine can be detected in the amniotic fluid. The concentration of carnitine in the amniotic fluid decreases between 10 and 40 weeks of gestation (48). Hahn and coworkers have proposed that this decrease may reflect the retention of carnitine by the fetal tissues (48). The plasma and red blood cell total carnitine levels of the human fetus decrease with increasing gestational age (49) while the skeletal muscle carnitine concentration increases (6). In premature infants, the plasma carnitine levels decrease between birth and three days of life. Maternal plasma carnitine levels from mothers giving birth prematurely are higher than those of mothers giving birth at full-term (50). Thus the premature infant does not benefit from continuing maternal transfer of carnitine in late gestation. Maternal transfer of carnitine may continue as an important source even after parturition. In humans, breast milk levels of total carnitine increase during the first week of lactation (7). Sow's milk is also well supplied with carnitine. Kerner and coworkers (11) reported levels of total carnitine as high as 370 nmol/ml in sow's colostrum. This seemed to contribute greatly to the carnitine status of the piglets as plasma levels increased twofold and liver levels increased fourfold after two days of suckling. One explanation of the importance of maternal sources of carnitine to the neonate may be that the neonate has limited carnitine biosynthetic capability. Human neonates appear to have limiting levels of the final enzyme in the carnitine biosynthetic pathway. Rebouche and Engle (8) reported that γ-butyrobetaine hydroxylase (4-trimethylaminobutyrate, 2 oxoglutarate:oxygen oxidoreductase (3-hydroxylating), EC 1.14:11.1) exhibited an age dependence. Infant levels were approximately 12% of the adult mean level. None of the other enzymes in the synthetic pathway demonstrated an age dependence. The presence of exogenous carnitine in the neonatal gut may stimulate the development of enzymes involved in carnitine metabolism. In rat pups, levels of carnitine acetyltransferase and carnitine palmitoyltransferase in the small intestine mucosal cells increase after birth (51). Carnitine acetyltransferase concentration in the nursing rat pup is higher in the proximal small intestine. The levels reach a peak between days 10 and 14 of life and then decrease with weaning (21 days) (51).

Carnitine and Neonatal Energy Metabolism

The human neonate must depend on energy from hepatic glycogen stores until its needs can be met by gluconeogenesis, fatty acid oxidation, and the supply of calories provided by feedings (52). Hepatic glycogen stores are depleted within the first 24 hours of extrauterine life. At this time fatty acids become the primary fuel of the infant's heart (52). L-carnitine will stimulate the oxidation of [^{14}C]-palmitate in newborn human adipocytes (7). Carnitine may stimulate gluconeogenesis in newborn tissue through its stimulatory effect on fatty acid oxidation. This may occur through increased levels of acetyl-CoA resulting from increased fatty acid oxidation (30). In newborn rats, the levels of carnitine and carnitine acetyltransferase in brown adipose tissue increase rapidly after birth (52). The levels of carnitine palmitoyltransferase in heart and liver homogenates from rat fetuses are low (52), as are the levels of carnitine palmitoyltransferase in premature human adipocytes (7). Besides the stimulatory effect of carnitine on fatty acid β-oxidation, there is also apparently an effect on lipolysis (53). L-carnitine increased glycerol release from subcutaneous adipose tissue from newborn but not adult humans. The mechanism of this effect remains unclear.

Neonates produce high levels of ketone bodies. Williamson (54) has suggested that the newborn produces ketone bodise in response to high levels of nonesterified fatty acids according to the following scheme:

MILK------>NEFA---->β-OXIDATION--->ACETYL-COA--->ACETOACETATE
FAT STORES---↑

The high levels of nonesterified fatty acids from either milk or adipose stores provide substrate for β-oxidation. The acetyl-CoA so produced is then used in the synthesis of ketone bodies. In neonatal rats, the plasma ketone body concentration increases tenfold during the first 24 hours postpartum (33). The ketone body levels decline gradually toward the time of weaning. The liver carnitine concentration in these animals follows the same pattern.

McGarry and Foster (35) have proposed that the increased hepatic carnitine levels stimulate ketone body production by stimulating carnitine acyltransferase. The enzyme stimulation and subsequent β-oxidation and ketone body production may also result from a hormonal (increased glucagon:insulin ratio) suppression of malonyl-CoA production, since malonyl-CoA is a potent inhibitor of carnitine acyltransferase (35). Ketone body production in the neonate is probably important in lowering the glucose requirement in several tissues, especially in the developing brain (54).

Carnitine and Neonatal Thermogenesis

Carnitine is required for the heat-producing oxidation of fatty acids in brown adipose tissue (24). In late fetal rats, brown fat accumulates subcutaneously administered DL- or L-carnitine more rapidly than heart or skeletal muscle tissue (22). Subcutaneously administered deoxycarnitine (an inhibitor of carnitine acyltransferase) prevents the normal increase in oxygen consumption of young rats upon exposure to 25°C to 30°C temperatures (22). In 18-day-old rats cold exposure increased both brown adipose tissue carnitine concentration and carnitine acyltransferase concentration (55). In human neonates, white adipose tissue contains adipocytes which behave more like brown adipocytes in rats (56). These cells have increased levels of carnitine palmitoyltransferase compared to adult cells (56). It has been proposed that these cells function like brown adipose tissue in non-shivering thermogenesis (56). These cells also respond to norepinephrine stimulation with an increase in oxygen consumption in a manner similar to the brown fat adipocytes of rats (53).

Carnitine and Neonatal Nitrogen Metabolism

Carnitine has been associated with improvement of or protective effects on certain aspects of nitrogen metabolism. Transient hyperammonemia is a problem in neonates, especially in low birth-weight infants (57). Carnitine may offer protection from the toxic effects of high ammonia levels. L-carnitine reduced mortality from an LD_{100} dose of ammonium acetate in mice (3). In mice with sustained hyperammonemia, carnitine treatment stimulated ureogenesis (3). The authors proposed that the increase in urea production was due to the carnitine-stimulated increased production of acetyl-CoA. The increased levels of acetyl-CoA in turn could stimulate increased production of n-acetylglutamate, a positive effector of carbamyl phosphate synthetase.

Carnitine may also be involved in improving nitrogen balance. Carnitine administration to minipigs receiving total parenteral nutrition resulted in improved nitrogen retention and decreased excretion of urea nitrogen (58). The improvement of nitrogen retention would be an important factor in maintaining growth in the neonate.

Other Aspects of Neonatal Metabolism

It has been proposed that carnitine has a role in the development of the proper phospholipids and surfactant in the fetal lung (4). Administration of L-carnitine to pregnant rats resulted in increased total phospholipids and increased dipalmitoylphosphatidylcholine in the fetal lungs. The rat fetal lungs from dams given carnitine had carnitine levels at day 20 of gestation equivalent to those of fullterm fetuses (22-23 days).

Carnitine Transport and Compartmentation

Introduction

The existence of a cellular transport (uptake and/or efflux) system for carnitine has been investigated in a variety of different tissues and species. Carnitine transport systems have been described for liver, kidney, heart, brain, and intestine in several species of experimental animals. Direct comparisons of these systems is impossible due to the variations in animal systems and methodologies utilized. A survey of these systems does, however, reveal certain characteristics which may lead to a better understanding of carnitine transport and compartmentation in the whole animal.

Carnitine Transport in Liver Cells

Christiansen and Bremer (59) have investigated carnitine transport in isolated rat liver cells. These researchers noted that up to 80% of the endogenous carnitine in the cells was lost during the isolation procedure. Carnitine transport into the isolated hepatocytes occurred against a concentration gradient. The transport carrier appeared to be a common carrier for both carnitine and its precursor (γ-butyrobetaine). The carrier exhibited a higher affinity (lower K_m) for γ-butyrobetaine than for carnitine. Transport of both carnitine and γ-butyrobetaine was inhibited by 2,4-dinitrophenol (DNP) suggesting an energy dependence. Several analogs of carnitine (choline, 1-dimethylamine-2-propanol, methylcholine) exerted inhibitory effects on the transport system. The finding of both a higher affinity for γ-butyrobetaine and release (efflux) of carnitine from the cells led these workers to propose that the precursor was transported into the liver cell, hydroxylated to carnitine, and released from the cell. French and coworkers (60) found that rat heart and kidney uptake of carnitine was stimulated by the steroid dexamethasone. No effect of the steroid on liver carnitine uptake was observed. These researchers proposed that increased heart and kidney uptake of carnitine might occur as a stress response. The same stress-related (steroid) stimulus might be a signal for increased hepatic synthesis or retention of carnitine.

Carnitine Transport in Heart Cells

Carnitine uptake has also been investigated in heart cells of both rat and human origin. Adult rat heart myocytes accumulated carnitine against a concentration gradient (61). Like the rat liver cells (59), the heart cells lost up to 69% of their endogenous carnitine as a result of the isolation procedure. The uptake was not sodium-dependent. Decreased calcium ion concentration in the media stimulated carnitine uptake. While the initial rapid uptake of carnitine was not inhibited by DNP, the slow, higher affinity system was inhibited. This suggested the existence of two systems, one a low affinity (diffusion) system and the other a high affinity, energy-requiring system. Inhibition of the system was seen with deoxycarnitine and acetylcarnitine. Both D- and L-carnitine were inhibitory at higher concentrations. Decreased uptake was also observed with both β-adrenergic agonists and antagonists and dibutyryl cAMP. Also, the pharmacological agents isoproterenol, propanolol, and lidocaine decreased the uptake of carnitine. An extensive series of investigations has been done on the carnitine transport system of a human heart cell line (Girardi heart cells CCL 27). Bohmer et al. (62) first suggested that these cells demonstrated active metabolism and uptake of carnitine. The system was temperature dependent and was inhibited by both the carnitine precursor γ-butyrobetaine and acetylcarnitine. In a similar study, Molstad et al. (63)

found that the carnitine uptake by these cells (CCL 27) could be reduced by inclusion of sulfhydryl blocking agents such as n-ethyl- maleimide and 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB, Ellman's Reagent). The transport was not related to the transport systems for amino acids, glucose transport, or dependent on $(Na^{+}K^{+})$ATPase. The uptake of L-carnitine was greater than that of D-carnitine. L-acetylcarnitine appeared to compete with L-carnitine for uptake. It was suggested that the carnitine transport system had two recognition sites and thus recognized compounds with both a trimethylamino group and a carboxylic group. In a subsequent report from this same laboratory (64), it was suggested that the transport of carnitine into the heart cells was dependent on new protein synthesis. This suggestion was based on the finding that the uptake of carnitine by these cells could be inhibited by cycloheximide. This same paper also included an electron microscopy study of the cells of the CCL 27 line, revealing that the cells were dedifferentiated, had no myofilaments, and in fact could no longer be considered typical heart muscle cells. Thus, further observations on the carnitine transport characteristics of these cells, while interesting and important contributions, could not be assumed to be representative of the cells in the whole animal. Molstad and Bohmer (65) later reported that prednisolone stimulated the uptake of carnitine by these cells. This effect was similar to that reported by French et al. (60) on the stimulatory effects of dexamethasone on rat heart carnitine uptake. This finding was significant in light of the fact that some patients with lipid storage myopathies and low intracellular carnitine levels had been treated with some success with prednisolone. Finally, Molstad (66) reported that there was rapid efflux of acetylcarnitine subsequent to uptake of carnitine by the CCL 27 cells.

Carnitine Transport in the Brain

A carnitine transport system has also been described for rat brain (44). This system is stereospecific for L-carnitine and is both sodium and temperature dependent. The system appears to be energy dependent. Carnitine is transported against a concentration gradient. Both GABA (γ-aminobutyric acid) and homotaurine (3-aminopropane sulfonic acid) inhibited the uptake of carnitine. This was of interest as earlier studies by these same researchers demonstrated that carnitine transport by kidney cortex was not inhibited by GABA or homotaurine (67). Thus the possibility of tissue specific carnitine transport characteristics does exist.

Carnitine Transport in Intestine

Carnitine transport in intestinal tissue has received increasing attention. Shaw and coworkers (68) investigated carnitine transport in rat duodenal, jejunal, and ileal everted rings. Intestinal segments from duodenum and jejunum but not ileum accumulated carnitine against a concentration gradient. The transport system was sodium dependent and could be inhibited by anoxia. Both D-carnitine and acetylcarnitine inhibited the uptake of L-carnitine. Interpretation of data from this study is limited, however, by the use of everted "rings" of intestine since no control of serosal uptake or efflux is controlled for in this system. Gross and Henderson (69) studied carnitine transport in perfused rat intestine. Uptake of L-carnitine occurred two times as fast as uptake of D-carnitine. The most rapid uptake was observed in the upper jejunal segment of intestine. L-carnitine uptake was saturable and could be inhibited by D-carnitine, trimetylaminobutyrate, and L-acetylcarnitine. Of particular interest was the finding of differences in the skeletal muscle accumulation of D- or L-carnitine administered in the intestine. L-carnitine in the muscle accounted for 29.5% of the L-carnitine radioactivity while D-carnitine only accounted for 5.3% of its administered radioactivity. The renal threshold for the L-carnitine was 80 µM; that of the D-carnitine was 30 µM. Thus the natural isomer was apparently preferentially retained in the body. The amount of radioactivity in the lymph was low, less than 0.25% of the initial dose after two hours. Sachan and Ruark (70) also concluded that the proximal small intestine was the most active in carnitine uptake. Gudjonsson and coworkers

(71) similarly investigated uptake of radioactive carnitine from the rat proximal small intestine. They noticed a slow appearance of radioactivity in the blood which was still increasing two hours after intestinal administration. Radioactivity was found in the bile but little (less than 0.02%) was found in the lymph. These researchers proposed that carnitine was absorbed by active uptake into the proximal intestinal mucosa where it was esterified and entered the portal circulation. From the portal circulation it was extracted by the liver and excreted into the biliary tree or released by the liver in response to an unexplained "intestinal signal." This same laboratory (72) more recently reported that the rat intestinal carnitine transport system was not related to the imino acid (proline) transport system. Again the apparent long retention and slow (at least eight hours) release of radioactivity by the intestinal mucosal cells were noted. These workers repeated similar experiments using human intestine biopsies (73) and found carnitine uptake occurring in the duodenum and ileum but not in the colon. Like the rat intestine system, carnitine uptake in the human intestine occurred against a concentration gradient, was sodium dependent, and was inhibited by both D-carnitine and L-acetylcarnitine. Finally, Gross et al. have investigated the uptake of carnitine by guinea-pig jejunal enterocytes (74). The uptake of L-carnitine by these cells is thought to be both temperature and sodium dependent. The

system could be inhibited by D-carnitine and by trimethylaminobutyrate. Ileal cells demonstrated one-half of the uptake of the jejunal cells. It was suggested that the intestinal carnitine uptake process might occur via facilitated transport as no energy dependence (actinomycin A sensitivity) was observed.

Carnitine Compartmentation

Carnitine transport and compartmentation in the whole animal have been investigated in the dog and the rat. In 1962, Yue and Fritz (75) studied the metabolism of D,L-methyl-[^{3}H]-carnitine in dogs. Intravenous administration of the label resulted in rapid disappearance from the plasma with subsequent appearance of label in the urine. There was no detectable uptake of radioactivity by the brain or by the red blood cells. Liver radioactivity peaked rapidly and then began to decrease by three hours post-administration. A small portion of radioactivity was found in the phospholipid fraction of the tissues. This fraction could not be identified but its chromatographic peak was similar to that of phosphatidylcholine, leading these workers to propose the conversion of a small amount of the labeled carnitine to "phosphatidylcarnitine." There was no apparent conversion of the carnitine to any catabolic products. Rebouche and Engel (76) also studied the compartmentation of carnitine in the dog. Their analysis was based on the assumption of a three compartment open system model. In their model the first compartment (pool "a") consisted of the extracellular fluid. It was assumed that this compartment supplied the other compartments with carnitine. The second compartment (pool "b") was made up of the cardiac and skeletal muscle. This compartment was characterized by slow uptake of label. The third compartment in this system was pool "c" which included the other tissues, most notably the liver and kidney. These workers observed no catabolism or further metabolism of the labeled carnitine in the muscle or urine. The feasibility of studying carnitine compartmentation in the whole animal, using analysis systems such as those proposed by Shipley and Clarke (77) was suggested by these workers. Several differences between rat and human carnitine metabolism such as the tissue sites of carnitine synthesis and the skeletal muscle to plasma ratio were overcome by the use of the dog model. Some problems were apparent with the dogs, however, the most critical being the observation that the animals did not appear to be in steady-state. Two groups of researchers have utilized the rat model to study carnitine compartmentation in the whole animal. Cederblad and Lindstedt (78) investigated the compartmentation of radioactive carnitine administered to rats yet noted confounding factors such as wide variations in the endogenous carnitine concentrations between individual animals and discrepancies between the calculated urinary carnitine elimination and the actual amount of radioactivity recovered. Brooks and McIntosh (79) also used the rat model and again observed serious problems with the maintenance of steady-state, especially in the liver where both influx of carnitine from the periphery and hepatic synthesis could result in increasing pool sizes. These workers assumed that the blood plasma was the reservoir of and only transport pathway for carnitine between the tissues; they also assumed that there would be no tissue contamination by the blood. One other difficulty to consider in carnitine compartmental analysis brought to light by this study was the problem of regional differences in carnitine concentration ("subcompartments") within a tissue. In an earlier study (80), Brooks and coworkers divided the rat tissues into three groups according to the behavior of the radioactive carnitine. Group one included the liver, kidney, spleen, adrenal, seminal vesicle, and prostate. This group of tissues demonstrated maximal accumulation of label within two hours. The second group included the heart, testis, and pancreas and showed maximal accumulation of label within two to 24 hours. The third group, brain and epididymal fat, showed little or no accumulation of label.

Summary

Carnitine transport systems have been proposed for several different tissues including liver, heart, brain, and intestine. Several characteristics, such as temperature dependence and inhibition by carnitine analogs, seem to be common to the different tissue systems. Other characteristics appear to be tissue specific. As Bremer (13) has proposed, the differences in carnitine uptake between the tissues may explain the variations in carnitine concentration throughout the body. Carnitine compartmentation has been studied to a lesser extent. Rapid appearance of intravenously administered label in the liver is one characteristic common to these studies, as is the lack of any apparent carnitine catabolism. The problems of investigation of carnitine compartmentation have included lack of maintenance of steady-state, variations between animals in endogenous carnitine levels, and incomplete recovery of radioactivity.

The Neonatal Piglet as an Animal Model

Introduction

The development of the neonatal piglet as a model for neonatal carnitine metabolism is based on the precedent set by previous research using the piglet as a successful model for investigating other aspects of infant metabolism. This review considers these previous uses of the piglet and the evidence supporting the development of this animal model for neonatal carnitine metabolism. Important metabolic conditions that must be controlled when using neonatal piglets as models for human infants are discussed. In addition, techniques for management, surgery, and stress reduction of piglets are discussed in terms of suggestions for maintaining viable piglets. Finally, successful investigations using colostrum-deprived piglets are reviewed with an emphasis on specific practices which the authors suggest increase the viability of these piglets.

Several researchers have proposed the use of the piglet as an animal model for investigations concerning human infant metabolism. As early as 1959, McCance and Widdowson (81) suggested that similarities existed between human and piglet neonatal metabolism. Several groups have proposed the piglet as a model for developmental fatty acid metabolism in man. Purvis et al. (82) reported that the fatty acid changes in developing piglet brain paralleled those of the human. He suggested the piglet as a model for human infant brain fatty acid content. The level of total brain fatty acid and of essential fatty acid increases from day 65 to day 114 of gestation. Clandinin et al. (83) found that fetal piglet brain and liver were capable of elongation-desaturation of fatty acids. Johansson and Karlsson (84) reported that the changes in serum lipids in both the neonatal and older piglet approaching adulthood parallel the pattern of changes in man. The LDL decreases with increasing gestational age while HDL increases. Total serum lipids, LDL, and HDL increase after birth. Anderson et al. (85) have found that neonatal minipigs fed an essential fatty acid (EFA) deficient diet from day 6 will develop signs of EFA deficiency by day 28 of life. These gross evidences of EFA deficiency included scaly skin, scabby tails, and exudative lesions.

Cooper (9) cited similarities such as the level of maturity at birth, anatomical similarities, susceptibility to hypothermia, limited thermal insulation, and increased metabolic rate in the first few days of life as support for the use of the piglet model for human pediatric research. Mayfield et al. (86) suggested the piglet as a model for human infant thermoregulation because of such similarities as the lack of a thick hair coat, a large surface area to mass ratio, and the ability to increase tissue insulation by peripheral vasoconstriction. Also like the human infant, the neonatal piglet may suffer long-term effects from hypothermia. Schneider et al. (87) found that small intestine tissue injury (necrosis, hemorrhage, shortened villi) occurred in 94% of neonatal piglets when the core body temperature was reduced by 4 ± 1°C for 4.5 hours. This intestinal damage, characteristic of neonatal necrotizing enterocolitis (NNEC), could be related to decreased gastrointestinal blood flow which is known to occur in the piglet during cold stress. Schneider et al. suggest that hypothermia and the resultant hypoxia of gastrointestinal tissue can result in ischemic injury and NNEC lesions in the neonates of both species. Schneider et al. also suggest that both species may benefit from a "protective" effect of breast milk ingestion in the lessening of such hypothermic effects. Thus the piglet may share features of thermoregulation with the infant. Both species are sensitive to long-term effects of hypothermia, especially when breast milk is not ingested.

Glauser (88) further pointed out the advantage of litter size of the pig, in that it might allow a statistically significant number of animals for an experiment from single litters. The piglet model also has the potential advantage of access to breeding records and known parentage of the progeny. Schneider and Sarett (89) suggested another advantage in that unlike rodent models, piglets could be utilized for nutritional studies from the day of birth on rather than from weaning on. This would more closely model the utilization of nutrients by the infant. Thus the use of the piglet as a model for investigating pediatric and infant metabolism has been promoted by several research groups and has received increased attention in recent years.

The piglet appears to be a good candidate for an animal model for neonatal carnitine metabolism. As is the case with the human infant, carnitine is most likely critical to the energy metabolism of the neonatal piglet. Carnitine levels in the skeletal muscle of the human fetus increase with increasing gestational age (8). A similar finding in the gestational piglet would lend support to the development of this animal model. Like the human infant, the piglet normally receives considerable amounts of carnitine in colostrum and milk. Kerner et al. (11) reported very high levels (370 nmol/ml) of total carnitine in sow's colostrum. While the heart and muscle tissue of the newborn piglet had considerable carnitine, the blood and liver had low levels of carnitine which increased by two- and fourfold, respectively, after 2 days of suckling. Thus it appears that the piglet and human neonate do share potential similarities in carnitine metabolism. Neonates of both species normally benefit from high levels of carnitine in the colostrum and milk. However, the preterm or sick human infant often receives no exogenous carnitine during a time when carnitine intake would normally be high. These infants, receiving no carnitine, often suffer from many metabolic abberrations (lipemia, decreased ketogenesis, poor gluconeogenic control) for which carnitine may be beneficial. Creation of a piglet model mimicking the situation of these infants would require colostrum deprivation and handfeeding of a carnitine-free diet formula. The successful development of techniques for raising and managing these colostrum-deprived piglets would likely result in a viable animal model for neonatal carnitine metabolism.

Importance of Environment and Management

Successful development of the piglet as an animal model for neonatal carnitine metabolism requires careful consideration of several unique characteristics of newborn piglet metabolism. Attention must be given to these points in order to assure the most efficient utilization of the piglet model. These include the potential differences in energy metabolism between the piglet and human infant and the existence of subpopulations of piglets within litters.

Several potential differences in metabolism between the human infant and the neonatal piglet may be overcome by careful control of the piglet's environment and dietary intake. The energy metabolism of the piglet, in terms of glycogen and lipid availability, would normally differ from the infant. Mellor and Cockburn (10) discuss neonatal piglet metabolism in comparison to the newborn lamb and human infant. For newborn mammals, the sources of carbohydrate energy will be body glycogen (liver and skeletal muscle) and colostral lactose, while the available fat will come from nonstructural body fat and the colostral lipid. The relative contribution of the sources and thus the primary source of metabolic energy will differ in the normal situation in these species. In contrast to the human infant, the neonatal piglet will normally have a large intake of colostrum on the first day of life. This may explain the difference in available liver glycogen stores between normal newborn piglets and infants (15% of available glycogen versus 40%). In contrast, piglets, in which shivering thermogenesis predominates, have the highest values for skeletal muscle glycogen stores. The availability of carbohydrate and not lipid will determine the period of time in which the well-being of the neonate will not be threatened. Even in the face of available lipid, depletion of glycogen stores can lead to hypoglycemia in the neonate. This will lead to a decrease in heat production and, unless the neonate is in a thermoneutral environment, hypothermia will follow.

In light of these metabolic characteristics of the neonatal piglet, Mellor and Cockburn propose several guidelines for the optimal use of piglets as animal models for the human infant. The first suggestion is the maintenance of thermoneutral conditions. An environment of thermal neutrality (32 to 38°C) will be advantageous for two reasons. First, this environment will mimic the human infant's birth environment. Secondly, thermal neutrality also will eliminate the changes in substrate utilization caused by the requirements for cold-induced thermogenesis. The use of the piglet's muscle glycogen will be limited until the liver glycogen stores are depleted, a situation similar to that normally occurring in the infant. The second suggestion Mellor makes is that colostrum intake be restricted in order to mimic the characteristically low early colostrum intake of the infant. Adherence to these guidelines should allow the piglet model to follow more closely the normal metabolism of the human infant.

Another critical point to consider is the potential experimental error to be derived by inclusion of runt piglets in normal piglet populations. The runt piglet is metabolically different from its siblings in terms of energy reserves, thermoregulation, and development. Hayashi (90) reported that "runt" piglets (less than 600 g body weight) demonstrated less of an increase in heat production than normal piglets when exposed to cold (32°C). It was suggested that this was partially due to the increased surface area to body weight ratio in the runt piglet. Aberle (91) has investigated the muscle myofiber differences between runt pigs (less than 850 g birth weight) and normal piglets. Runt pigs have a lower proportion of type I fibers in the deep semitendinosis muscle. Aberle speculates that this may be the result of a restriction in prenatal myofiber hyperplasia. Other researchers (90) have reported lower levels of respiratory enzymes in the muscles of runt piglets. These findings can be taken as further support for limiting the use of runt piglets as animal models or considering them as a separate population of animals.

Mellor and Cockburn (10) propose that normal piglets may be the best non-primate animal model for preterm or growth retarded infants, particularly when the environmental and experimental conditions are carefully chosen. The importance of maintaining the animals in an environment of thermal neutrality to mimic the human infant in terms of substrate utilization (by limiting muscle glycogen use until liver glycogen is depleted) and in terms of birth environment is stressed. A final critical point is the exclusion of runt piglets from the experimental population.

Aside from consideration of the unique metabolic characteristics of the newborn piglet, certain techniques and practices have been used to improve the viability of the newborn and early-weaned piglet. These include sound management practices during birth of the piglets.

Several researchers have investigated the possibility of early weaning of piglets as a means of increasing the productivity of breeding sows by allowing more litters to be born per year (92). It has also been suggested that the development of techniques for successful rearing of early weaned piglets could reduce piglet mortality by removing disadvantaged piglets from the sow to an environment where their chances of survival would be increased. Artificial rearing would also be helpful in the case of litters too large for a sow to successfully feed (92). Pre-weaning mortality still claims between 20 and 25% of the piglets born in this country, even with the advanced management systems in practice today (93). Of these losses, 80% occur either during parturition itself or during the first week of life. Sound management practices, such as attendance at farrowing and the clamping of the umbilical cord can reduce some of these losses (93). Labor induction techniques such as the use of prostaglandin F2a and oxytocin are also helpful as the time of farrowing can be controlled and personnel can be scheduled ahead of time to attend the farrowing (94,95). This is of critical importance for the acquisition of colostrum-deprived piglets. The response of older sows to labor induction practices is generally more predictable than that of gilts (95). Both the length of time and the variability of time from injection of oxytocin to the start of parturition can be decreased with an increased dose. Higher doses of oxytocin, however, increase the requirement for manual aid during farrowing, possibly due to overstimulation and subsequent exhaustion of the uterus (94,95). Thus management practices such as labor induction and attendance at farrowing can greatly decrease the mortality of newborn piglets.

The development of anesthesia and surgical techniques for newborn piglets has been very limited. Most work has been performed on older, weaned pigs. Hoyt et al. (96) caution that swine lack the tractability, anatomical attributes, and anesthetic response of dogs. He suggests a combination of ketamine and azaperone for surgical techniques such as catheterizations. Schieber et al. (97) have investigated the response of newborn piglets to isoflurane and halothane. A low margin of safety was observed for piglets exposed to isoflurane. This response is thought to correspond to the newborn human hemodynamic response of mild to severe bradycardia (97). Coalson et al. (98) have made similar observations and stresses the importance of maintaining light maternal anesthesia when deriving piglets by cesarian section.

Venous catheterization techniques have been developed for larger piglets (99,100). Mustard et al. (100) successfully used Silastic tubing catheters for a blood sampling experiment. A prophylactic dose of antibiotic prior to catheter placement was thought to aid in the prevention of infection of the incision sites (100). A loss of catheter patency was observed when the end of the tubing was found to rest against the wall of the vein (100).

Thus it appears that caution is warranted in exposing newborn piglets to anesthesia. Use of a vaginal delivery system avoids exposure of the piglets to maternal anesthesia during a cesarean section. Ketamine would seem to be a good choice of anesthesia, especially when used in combination with other drugs such as azaperone. Antibiotic use can be helpful in preventing infection of the catheterization site. Careful insertion of catheters and choice of both venous site and tubing gauge are important in maintaining catheter patency.

Another factor that must be considered when investigations using piglets are planned is the level of stress to which the animal will be exposed. The effects of various types of stress on swine have been investigated

(101). Stressing pigs can be detrimental to nitrogen balance, metabolism, and growth (101). Swine seem to be particularly sensitive to stressful handling by humans (101). Barnett and Hemsworth (101) found that gilts exposed to pleasant human contact (stroking or scratching) had significantly improved growth performance and pregnancy rate (87.5% versus 33.3%) over gilts exposed to unpleasant handling (slapping). Stressful handling also resulted in a chronic elevation in corticosteroid levels. Barnett and Hemsworth recommend the avoidance of a "poor human-animal relationship" in both swine production and experimentation. Similarly, Takahashi (55) compared the physiological stress of conventional venipuncture blood sampling techniques to use of in-dwelling catheters in 40 day old piglets. Plasma 11-hydroxycorticosteroid, glucose, white blood count, hematocrit, body temperature, and heart rate were elevated in the venipuncture bled but not in the catheterized piglets. Corticosteroid levels remained elevated for more than one hour in the piglets bled by conventional means. Thus, interactions with piglets should be as non-stressful and pleasant to the animal as possible, in order to avoid disturbances in metabolism. The use of surgically inserted, in-dwelling catheters for dosing and sampling would be less stressful than traditional venipuncture or, for gastric dosing, gavage techniques. This is probably of critical importance concerning carnitine metabolism since stress resulting in elevation of steroid hormones may affect carnitine levels in the tissues (60).

Colostrum-Deprived Piglets

Attempts at raising colostrum-deprived piglets for meat production uses have been made (98,102). Most of these investigations have used a germ-free, sterile technique to avoid the problems of disease in the immune-compromised piglets. Coalson et al. (98) derived piglets from cesarean sections and transferred them to sterile incubators. The maternal dose of anesthesia (fluorothane) was kept as light as possible to decrease piglet recovery time. These piglets were fed a formula based on fresh whole cow's milk. The survival rate to three weeks of age was 89% in this study. Varley et al. (102) utilized similar techniques but used vaginally delivered piglets. The piglets were delivered directly into a sterilized plastic bag and transferred to the sterile incubators. The piglets in this study also consumed cow's milk at a rate of one feeding per hour. The intake of milk was restricted after it was observed that feeding to appetite resulted in diarrhea. Such diet restriction unfortunately also decreased the rate of live weight gain in the piglets. The survival rate per litter ranged from 55 to 98%. Varley stresses the importance of an "all in/all out" policy of not introducing new piglets into an already occupied piglet facility in using facilities for colostrum-deprived piglets. It was thought that thorough disinfection between litters and the avoidance of introducing piglets from new litters when the room is occupied helped to reduce mortality from disease. Both of these researchers used individual incubators and maintained the room temperature at 33°C or higher for at least the first 72 hours postpartum. Thus, successful attempts at raising colostrum-deprived piglets utilized sterile techniques, individual incubators, and temperatures approaching or within the thermal neutrality range. The dietary formulas were based on cow's milk and were apparently well-tolerated, especially when intake was frequent and the amount was restricted. Cow's milk, like sow's milk and human breast milk, contains high levels of carnitine and could not be fed where a carnitine deprived piglet was desired. Also, the feeding of breast milk or milk-based formula to preterm or sick neonates is uncommon and thus a neonatal piglet model for this population would ideally not consume a milk-based formula. Weight gain in the previously attempted investigations utilizing colostrum-deprived piglets was not as rapid as desired. This is a problem common to most early weaning regimens in piglets. Leece (92) has called this phenomenon the "post-weaning check." From a production standpoint this is clearly a concern with early weaning practices. When colostrum-deprived piglets are used for neonatal models, however, this reduced growth rate could be considered advantageous since a reduction in growth rate would make the piglet more comparable to the human neonate. Indeed, Varley recommends the colostrum-deprived piglet as an attractive model for neonatal nutrition (102).

Summary

The neonatal piglet and the human infant share many metabolic similarities such as developmental changes in fatty acid secretion patterns and sensitivity to cold stress. Carnitine is likely critical to the metabolism of both the neonatal piglet and the human infant. Thus the neonatal piglet, deprived of colostrum and carnitine feeding, is a potentially useful animal model for investigations of carnitine metabolism in the sick or preterm human neonate. Based on the previously reported research in the literature, the development of a neonatal piglet model for investigations of human infant carnitine metabolism will require careful control of the piglets' environment and utilization of specific management practices to ensure the viability of the piglets for experimentation. Ideally, the neonatal piglet should be maintained in an environment of thermal neutrality both to mimic the human infant's birth environment and to limit the utilization of energy substrates for thermogenesis. Runt piglets should be considered as a separate subpopulation due to developmental and metabolic differences. Management practices, such as use of labor induction and attendance at farrowing, are suggested as a means to improve the survival of neonatal piglets. The viability of the piglet model may be greatly improved by avoidance of unnecessary stress to the animal. The neonatal piglet's potential as an animal model for the study of infant carnitine metabolism should be greatly increased by the utilization of these suggestions from the literature.

CHAPTER 3

TOTAL CARNITINE LEVELS IN THE GESTATIONAL PIGLET

Introduction

Similarities in physiology and metabolism indicate that the neonatal piglet is an appropriate animal model for investigations of human neonatal metabolism. To support the use of the neonatal piglet as an animal model for human neonatal carnitine metabolism, it is necessary to establish that the neonates of both species begin extrauterine life in a similar carnitine status. Colostrum and milk of both humans and swine contains high levels of carnitine (1). In the human fetus, carnitine levels increase in skeletal muscle with increasing gestational age (6), while plasma and red blood cell carnitine levels decrease with increasing gestational age (49). A finding of a similar pattern in carnitine accretion during gestation in the piglet fetus would suggest that both neonatal humans and neonatal piglets begin extrauterine life in comparable carnitine status. This would aid in the development of the neonatal piglet as a model for investigating human neonatal carnitine metabolism. Thus, this experiment was designed to investigate the pattern of carnitine accretion in the fetal piglet throughout gestation. The levels of carnitine in fetal lung, liver, kidney, and intestine which have not been measured previously in the human fetus were measured in order to identify similarities in accretion patterns between the tissues. In addition, the levels of carnitine in the fetal piglet amniotic and allantoic fluids were measured.

Materials and Methods

Animals

All swine used in this experiment were from the Department of Animal Science Research Breeding Herd and were provided through a collaborative research agreement with Dr. Fuller W. Bazer in the Department of Animal Science. Piglets were taken on days 60, 78, 85, 90, 97, 103, 110, and 112 of gestation by cesarean section from domestic crossbred (Yorkshire x Hampshire x Duroc) gilts. The gilts were anesthetized initially with 1 g thiamylal sodium (5 percent solution) injected via an ear vein. Anesthesia was maintained by halothane. A midventral laparatomy was performed to expose the uterus. The fetuses were removed from the uterus, assigned an identification number, and transported to the laboratory for tissue sampling. Fetal blood was collected from the umbilical vein and maternal blood was collected from a uterine vein. Allantoic and amniotic fluids were collected from the allantoic and amniotic sacs with an 18 gauge needle and syringe.

Fetuses were covered with ice until dissection and tissue collection were completed. Heart, lungs, liver, small intestine, kidneys, and skeletal muscle (gastrocnemius region) samples were removed from fetuses and placed in individual plastic weighing boats on ice. Heart ventricles were trimmed of excess tissue, cut open, and swirled gently in chilled saline (0.9% NaCl) to remove blood. Lungs, kidneys, and skeletal muscle were trimmed of excess tissue and rinsed in saline. Intestinal tissue was cut lengthwise and rinsed with saline to remove the intestinal contents. Whole livers were perfused with saline (via the intact vasculature) to clear any blood. All tissue samples were blotted to remove excess moisture, placed in prelabeled plastic vials, and stored at -80°C. Fetal and maternal blood samples were centrifuged (Model TJ-6, Beckman Instruments, Palo Alto, CA) at 3,000 x g for 15 min at 4-6°C to separate plasma and red blood cells. Plasma was frozen. The red blood cells were washed twice with saline and frozen.

Tissue Analyses

Tissue homogenates of 250 mg (liver, lung) or 150 mg (all other tissues) were prepared in 3 ml of distilled water, using a Ten Broeck homogenizer (Fisher Scientific, Springfield, NJ). The carnitine concentrations of the tissue homogenates, plasma, red blood cells, and allantoic and amniotic fluids were measured by the method of Cederblad and

Lindstadt (103) (Appendix A). Protein concentration was determined by the micro-biuret method of Itzhaki and Gill (104) (Appendix A). The method of Lilienthal et al. (105) (Appendix A) was used for the solubilization of noncollagen protein. Hemoglobin concentration of the red blood cells was measured using a Fisher Diagnostic Kit (Fisher Diagnostics, Orangeburg, NY) based on a modified Drabkin method (106) (Appendix A).

Statistical Analyses

Statistical analyses of data were performed using the RS/1 Integrated Data Analysis System (Bolt Beranek and Newman, Inc.) for the Digital Professional 350 (Digital Equipment Corporation, Maynard, MA). Sample populations were subjected to the Wilk-Shapiro test of normality. Normal populations were tested for equal or unequal variances with an F-test for variance ratio. A T-test for either equal of unequal variances was then performed. In the case of non-normal populations the Ansari-Bradley test for equal dispersions was used, followed by the Mann-Whitney test for unpaired samples with equal dispersions. Correlations, when performed, were made by calculating R and R-squared of two sample populations using the RS/1 software (107).

Results

Fetal red blood cell total carnitine concentrations decreased from 90 days gestation to 112 days gestation

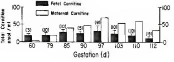
(Figure 3-1). Fetal plasma carnitine levels decreased from 97 days to 112 days gestation (Figure 3-1). The ratio of maternal to fetal plasma carnitine concentrations increased steadily from 0.2 at 60 days to 3.7 at 112 days. The ratio of maternal to fetal red blood cell carnitine did not follow the same pattern with values remaining below 0.5 until after day 103 and then increasing to a mean of 0.69 in one litter and 1.56 in a second litter at 112 days.

Fetal skeletal muscle total carnitine levels increased with gestational age (Figure 3-2). While cardiac muscle carnitine concentrations (Figure 3-2) also increased between 60 and 112 days gestation, the levels reached peak concentrations earlier than in the skeletal muscle.

Total carnitine concentrations in the fetal liver, kidney and intestine (Figure 3-3) reached a peak at 90 days gestation. Fetal lung total carnitine followed a similar pattern with a high plateau from 79 to 97 days and a low plateau from 103 days until fullterm (Table 3-1). Lung total carnitine per mg noncollagen protein patterns correlated well with kidney (R=0.7571), intestine (R=0.8275), liver (R=0.7747), and red blood cell levels (R=0.8925).

The levels of total carnitine in allantoic and amniotic fluids at different days of gestation are shown in Tables 3-2 through 3-5. Allantoic fluid total carnitine (nmol/ml) increased from 25 to 30 days gestation. A similar pattern

Figure 3-1: Fetal and maternal plasma total carnitine concentrations (top graph) at various days of gestation. Fetal values at 110 and 112 d are less than those at 97 d ($p<0.01$). Bars represent Means ± S.D. Numbers in parentheses represent the number of piglets in each sample population. Fetal and maternal red blood cell total carnitine concentrations (bottom graph) at various days of gestation. Fetal values at 97, 103, 110 and 112 d are less than those at 90 d gestation ($p<0.01$). (Note: Maternal red blood cell sample from 110 d gestation was lost in preparation).


Fetal Carnitine
Maternal Carnitine
Total Carnitine nmol / ml
100
50
0
100
50
0
(3)
(10)
(10)
(5)
(9)
(10)
(10)
(18)
60
79
85
90
97
103
110
112
Gestation (d)

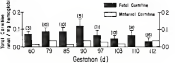
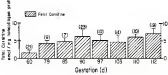

Fetal Carnitine
Maternal Carnitine
Total Carnitine nmol / mg hemoglobin
0.2
0.1
0.0
0.2
0.1
0.0
(3)
(10)
(10)
(5)
(11)
(10)
(8)
(16)
60
79
85
90
97
103
110
112
Gestation (d)

Figure 3-2: Skeletal muscle total carnitine concentrations (top graph) at various days of gestation in the piglet. Values at 60 d gestation were lower than values at 79, 85, 90, 97, 103, 110, and 112 d gestation ($p<0.01$). Cardiac muscle total carnitine concentrations (bottom graph) at various days of gestation. Value at 60 d gestation was lower than value at 112 d gestation. Bars represent Means ± S.D. Numbers in parentheses represent the number of piglets in each sample population.


Fetal Carnitine
Total Carnitine
nmol / mg noncollagen protein
10
5
0
(21)
(9)
(7)
(23)
(10)
(11)
(10)
(19)
60
79
85
90
97
103
110
112
10
5
0
Gestation (d)

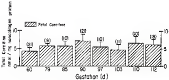
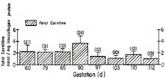
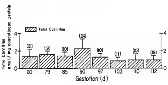

Fetal Carnitine
Total Carnitine
nmol / mg noncollagen protein
10
5
0
(21)
(9)
(10)
(21)
(10)
(11)
(10)
(18)
60
79
85
90
97
103
110
112
10
5
0
Gestation (d)

Figure 3-3: Liver total carnitine concentrations (top graph) at various days of gestation. Value at 90 d is greater than values at 60, 79, 85, 97, 103, 110, and 112 d ($p<0.01$). Kidney total carnitine concentrations (middle graph) at various days of gestation. Value at 90 d is greater than values at 60, 79, 85, 97, 103, 110, and 112 d ($p<0.01$). Intestine total carnitine concentrations (bottom graph) at various days of gestation. Value at 90 d is greater than values at 60, 79, 103, 110, and 112 d ($p<0.05$). Bars represent Means ± S.D. Numbers in parathases represent the number of piglets in each sample population.


Total Carnitine
nmol / mg noncollagen protein
Fetal Carnitine
6
4
2
0
(21)
(9)
(10)
(24)
(10)
(10)
(10)
(19)
60
79
85
90
97
103
110
112
Gestation (d)


Total Carnitine
nmol / mg noncollagen protein
Fetal Carnitine
4
2
0
(21)
(9)
(10)
(24)
(10)
(11)
(10)
(19)
60
79
85
90
97
103
110
112
Gestation (d)

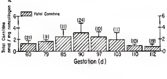

Total Carnitine
nmol / mg noncollagen protein
Fetal Carnitine
6
4
2
0
(21)
(9)
(11)
(24)
(10)
(11)
(10)
(19)
60
79
85
90
97
103
110
112
Gestation (d)

Table 3-1
Lung Total Carnitine per mg Noncollagen Protein

Gestation (days)	n	Mean ± S. D.	Range
79	9	0.97 ± 0.21^{A}	(0.68-1.17)
85	10	1.12 ± 0.21^{A}	(0.86-1.46)
90	12	1.22 ± 0.55^{A}	(0.61-2.45)
97	10	1.02 ± 0.47^{A}	(0.62-2.14)
103	11	0.47 ± 0.25^{B}	(0.15-0.89)
110	10	0.59 ± 0.35^{B}	(0.25-1.46)
112	19	0.45 ± 0.26^{B}	(0.00-0.93)

Note: Values within a column with different superscripts are significantly different.

Table 3-2
Allantoic Fluid Total Carnitine per ml

Gestation (days)	n	Mean ± S. D.	Range
79	8	99.0 ± 69.6^{A}	(44.8-261.1)
85	5	115.3 ± 32.9^{A}	(71.3-176.9)
90	15	119.5 ± 121.5^{A}	(23.0-417.6)
97	2	72.2 ± 22.6*	(56.7-88.6)
103	10	54.6 ± 17.7^{B}	(30.2-92.4)
110	1	50.3*	
112	13	38.5 ± 27.8^{B}	(0.0-98.8)

Note: Values within a column with different superscripts are significantly different.

*Excluded from statistical comparisons due to small sample size.

Table 3-3
Allantoic Fluid Total Carnitine per mg Noncollagen Protein

Gestation (days)	n	Mean ± S. D.	Range
79	8	$21.07 \pm 23.95^{A,C}$	(7.17-79.68)
85	5	11.73 ± 3.07^{A}	(7.31-15.72)
90	13	21.67 ± 13.26^{C}	(3.99-44.23)
97	2	8.35 ± 3.49*	(4.83-11.81)
103	10	5.09 ± 3.60^{D}	(1.00-12.08)
110	1	1.29*	
112	7	2.57 ± 0.92^{D}	(1.41-3.66)

Note: Values within a column with different superscripts are significantly different.

*Excluded from statistical comparisons due to small sample size.

Table 3-4
Amniotic Fluid Total Carnitine per ml

Gestation (days)	n	Mean ± S. D.	Range
79	10	16.1 ± 4.5^{A}	(11.9-23.3)
85	8	12.9 ± 3.3^{A}	(9.6-19.6)
90	11	21.3 ± 5.3^{B}	(14.1-34.6)
97	6	11.3 ± 3.7^{A}	(8.3-18.5)
103	11	11.4 ± 4.1^{A}	(7.0-19.5)
110	8	6.5 ± 3.2^{C}	(4.2-13.7)
112	11	6.4 ± 1.8^{C}	(4.0-9.9)

Note: Values within a column with different superscripts are significantly different.

Table 3-5
Amniotic Fluid Total Carnitine per mg
Noncollagen Protein

Gestation (days)	n	Mean ± S. D.	Range
79	10	7.66 ± 2.14^{A}	(4.48-10.35)
85	7	6.45 ± 2.45^{A}	(3.86-10.89)
90	12	11.67 ± 9.79^{B}	(5.73-42.14)
97	4	$13.28 \pm 4.54^{B,D}$	(9.27-19.80)
103	11	$13.83 \pm 6.98^{C,D}$	(1.00-20.89)
110	10	$6.17 \pm 3.64^{A,D}$	(1.92-11.38)
112	12	4.57 ± 2.16^{D}	(2.17-9.50)

Note: Values within a column with different superscripts are significantly different.

was observed for amniotic fluid total carnitine levels; however, samples for 25 and 60 days gestation were not available. The patterns of total carnitine in these fluids, when expressed as nmol/ml, correlated well (R=0.9277). This pattern was similar to that of the fetal kidney total carnitine levels (R=0.9103).

When the total carnitine levels in the fetal fluids were expressed as nmol total carnitine per mg noncollagen protein, the similarities between the two fluid's patterns were diminished. Amniotic fluid levels peaked at between 90 and 103 days and then decreased, while allantoic fluid levels peaked at 79 and 90 days and then decreased to full term (112 days).

Discussion

If the neonatal piglet (both preterm and term) is a valid animal model for the neonate, the profile of tissue carnitine concentrations during gestation must be similar. The patterns observed in the fetal piglets' plasma and red blood cell carnitine concentrations are similar to those for human fetuses and neonates (49). Both species show decreases from approximately 90 days (corresponding to 30 weeks human gestation) to term. The similar patterns of carnitine accretion in fetal red blood cell, plasma, liver, kidney, intestine, and lung suggest that these tissues represent a metabolic compartment for carnitine. The low maternal to fetal plasma carnitine ratio in early gestation may reflect maternal to fetal transfer of carnitine. The increase in this ratio in late gestation could reflect a decrease in this maternal to fetal transfer. The decreased carnitine levels in the tissues of this group (liver, intestine, kidney, lung) detected in this study support this hypothesis. While the role of carnitine in the fetal and neonatal lung has not been established, some researchers have reported that carnitine may have an influence on fetal lung weight and maturation (4,5). Lohninger et al. (4) found that carnitine administration to pregnant rats resulted in increased total phospholipid content of the fetal rat lungs relative to controls. Thus the accumulation of carnitine by this fetal tissue may be important in the lung maturation process.

Both human and piglet fetuses are surrounded by amniotic fluid in utero. This fluid apparently arises from the amniotic membrane surrounding the fetus in both species (108,109) although the exact origin of this protective fetal fluid is unclear. The piglet and human fetus may absorb amniotic fluid through both the gastrointestinal tract and the lungs. In the fetal piglet, an additional fluid reservoir is formed by the allantoic sac and its fluid contents (109). This sac forms from an outgrowth of the fetal hindgut (109). The allantoic fluid may be of maternal origin through the allantoic membrane (109). This fluid is rich in several nutrients and may exist as a nutrient reservoir for the developing fetus (109). In humans, amniotic fluid carnitine levels decrease between 10 and 40 weeks of gestation (48). Hahn et al. (48) proposed that this decrease reflected uptake of carnitine by the fetus. The carnitine levels of both the amniotic and allantoic fluids of the piglet fetuses peaked at approximately 90 days gestation and then decreased. This pattern, like that observed in the human amniotic fluid, may reflect fetal uptake of carnitine or decreased maternal transfer of carnitine. The similarities in the accretion patterns in the intestine, lung, and the fetal fluids could indicate that transfer of carnitine from the fluids to these fetal tissues occurs during gestation. The decrease in fetal blood and kidney carnitine levels in late gestation could also reflect a decreased maternal transfer of carnitine through these fetal fluids.

The observation of increasing skeletal muscle carnitine concentrations from 60 days (<26 weeks human gestation) to 112 days (38 weeks human gestation) determined in this study is similar to what occurs in humans (6). In human fetuses and infants, skeletal muscle carnitine concentration correlated positively with gestational age (6). Cardiac muscle carnitine concentration also increases, but appears to reach a maximum concentration earlier. This may reflect a greater or earlier need for carnitine in the fetal heart. The similarities in carnitine accretion patterns in the two muscle tissues suggest that they represent a second metabolic compartment of carnitine. The fact that these two tissues maintain their maximal levels while plasma, red blood cell, liver, kidney, intestine, lung, and fetal fluid levels decrease further suggests these tissues form a separate metabolic compartment. It is possible that by 90 days (30 weeks human) gestation the fetus has obtained a critical minimum level of carnitine in the muscle "stores." The 90 day gestational age corresponds to a human gestational age above which neonatal survival increases greatly.

The neonatal piglet is a good experimental model for the investigation of carnitine requirements in human neonates due to the many similarities in anatomy and physiology between the two species. Carnitine levels in piglet plasma, red blood cells, skeletal and cardiac muscle, liver, kidney, intestinal tissue, lung, and fetal fluids were studied. Results suggest the existence of a two-compartment system: one for red blood cells plasma, liver, kidney, intestine, and lung which show decreasing carnitine levels with increasing gestational age, and a second compartment for skeletal and cardiac muscle showing increasing levels of carnitine with increasing gestational age. The importance of carnitine in lipid metabolism and thermogenesis makes carnitine a critically important nutrient for the neonate. Carnitine levels increase in the muscle tissue of the piglet from 90 days of gestation to birth, which relates to the increase in muscle carnitine concentrations from the corresponding gestational age of 30 weeks to term in the neonate. Survival rates for the neonate improve at greater than 30 weeks suggesting a possible relationship to carnitine metabolism.

Thus, this investigation lends further support for the development of the neonatal piglet as an animal model for investigating human neonatal carnitine metabolism. This has been accomplished by establishing that the patterns of carnitine accretion during gestation in the blood and skeletal muscle are similar in both species. The patterns of carnitine accretion in the other tissues such as liver, kidney, intestine, lung, and fetal fluid which have not been measured in the human fetus differ from the pattern in the cardiac and muscle tissue. The difference in accretion pattern may be related to the existence of separate tissue compartments for carnitine. This can be further investigated with the development of a viable neonatal piglet model for investigating carnitine metabolism.

CHAPTER 4

AN INITIAL INVESTIGATION INTO THE USE OF THE NEONATAL COLOSTRUM-DEPRIVED PIGLET AS A MODEL FOR NEONATAL CARNITINE METABOLISM

Introduction

Carnitine is involved in several aspects of metabolism important to the neonate, including fatty acid β-oxidation and ketogenesis (1). Due to its role in these areas of metabolism and a potential role in gluconeogenesis, branched chain amino acid degradation, and ammonia metabolism, carnitine supplementation to premature or sick human neonates is under consideration. These infants represent a population of neonates who normally receive no exogenous carnitine, in contrast to healthy neonates receiving breast milk or carnitine supplemented infant formulas. Two potential routes of carnitine supplementation to neonates are the intravenous and intragastric (antral) routes. In order to devise an optimal supplementation method it would be ideal to have an understanding of neonatal metabolic handling or compartmentation of carnitine administered by these two routes. Information about neonatal carnitine metabolism and compartmentation is needed but realistically cannot be gained by direct experimentation on human infants. A full understanding of the infant's handling of carnitine would require extensive tissue sampling. Ideally, samples would be collected from the many potential tissue pools of carnitine, including blood, liver, kidney, gastrointestinal tract, lung, skeletal and cardiac muscle, and other tissues. The use of radiolabeled carnitine to trace the tissue uptake of carnitine would provide valuable information about carnitine transport and compartmentation in the neonatal body. Clearly, the undertaking of such investigations on human infants would be both impractical and unethical.

The development of an experimental animal model for use in studies concerning neonatal carnitine metabolism and neonatal nutrition in general is therefore necessary. This experiment was designed to ascertain the viability of the neonatal colostrum-deprived piglet as an animal model for investigations of neonatal nutrition and specifically neonatal carnitine metabolism. The primary goal of this study was to determine if methods and procedures allowing experimental use of viable colostrum-deprived piglets could be developed. Procedures would be needed to care for, feed, and sacrifice the piglets. In addition, procedures for surgical implantation of gastric and intravenous catheters would be required in order to investigate the tissue uptake of tracer carnitine from two routes of administration. Finally, procedures for analyzing both the endogenous carnitine levels and the levels of radioactivity resulting from tracer administration would be needed.

Materials and Methods

Animals

All procedures involving animals in this study met with prior approval by the All University Committee on the Care and Use of Laboratory Animals. The piglets used in this experiment were the result of three-way crossbreeding (Duroc x Yorkshire x Hampshire) of domestic swine in the University of Florida Research Breeding Herd, provided through a collaborative research agreement with Dr. Fuller W. Bazer in the Department of Animal Science. Piglets were delivered on day 112 of gestation (fullterm equals 113 to 114 days) using prostaglandin $F2_{\alpha}$ and oxytocin induced labor (94,95). Prostaglandin $F2_{\alpha}$ (12 to 15 mg) was injected at 7:30 AM on day 111 of gestation. At 9:00 AM on day 112 of gestation, the gilts or sows received an intramuscular dose of 20 IU of oxytocin. This labor induction procedure allowed control over the time of farrowing in order to ensure that the piglets were hand-delivered, ingested no colostrum, and could be rapidly transferred to the laboratory following birth. The piglets were delivered into clean dry towels and were rubbed dry. The umbilical cord was tied with umbilical tape and then severed with scissors. The piglets were assigned a number according to birth order. The time of birth, condition at delivery, sex, and color were recorded on the piglet medical chart. While awaiting transport, the piglets were placed in boxes and warmed by a heat lamp. As quickly as possible, the animals were transported to the Piglet Neonatal Intensive Care Unit (PNICU) in the animal facility in the Food Science Building.

Piglet Neonatal Intensive Care Unit Procedures

The PNICU room temperature was maintained at 34 to 36°C. The humidity was maintained at at least 40% humidity. Prior to the admission of piglets, the room was scrubbed thoroughly and all surfaces were swabbed with a disinfectant solution. Personnel entering the PNICU were required to wash from their fingertips to elbows at a wash station located at the sink in an adjacent room. Upon admission to the PNICU, the piglets were weighed and had their milk teeth clipped. Each piglet received a prophylactic dose of a broad-spectrum antibiotic injected intramuscularly (cefazolin, 100 mg per kg body weight). Piglets were then assigned to individual plexiglass incubators. The incubators were constructed of clear plexiglass and measured 14 x 14 inches in area and 17 inches in height. The floor of the incubators was covered with disposable paper liners. A clean, soft towel was provided as bedding for the piglets.

Nutritional Support of Piglets

Beginning on Day One of the experiment (Day One equals day 112 gestation), piglets were handfed 25 to 30 ml of a sterile piglet formula every two hours until four hours before surgery on Day Two. This formula was based on a commercial carnitine-free soy based infant formula. (The composition of the formula is listed in Appendix B.) On Day Two, during the surgery and recovery period, the piglets received intravenous fluid (5% dextrose, 1/2 normal saline) through the internal jugular catheter at a rate of 5 to 7 ml per hour. When the piglets had recovered sufficiently from anesthesia, the intravenous drip was discontinued and feedings of the piglet formula (25 to 30 ml every two hours) were resumed. On Day Three the piglet formula was fed at a rate of 40 ml every two hours until 6:00 PM, when the volume was increased to 60 ml every two hours. This regimen continued on Day Four until four hours prior to the start of the tracer carnitine uptake experiment.

Surgical Procedure

Surgery to insert intravenous and intragastric catheters was performed on Day Two, beginning at 7:00 AM. The piglets were weighed and the dosage of anesthesia was calculated based on body weight. Initial anesthesia was acheived by intramuscular injection of ketamine (75 mg per kg body weight). When adequate anesthesia was obtained the piglets were placed supine on a heating pad. The right neck was prepped with an iodophor solution. A 2 cm longitudinal incision was made on the medial border of the right sternocleidomastoid muscle. By blunt dissection using a hemostat the right internal jugular vein was isolated. A proximal ligature (triple knot) of 3-0 silk was placed around the vein and a distal ligature was looped around the vein. A small venotomy was made with venotomy scissors. A 20 gauge (0.03 inches internal diameter, 0.065 inches external diameter) silastic catheter was inserted to the level of the superior vena cava (approximately 7 cm). Patency was confirmed using a heparinized syringe and the catheter was then secured with a 3-0 silk ligature placed distally. The catheter was then tunneled subcutaneously to exit between the scapulae. An intravenous drip of acetylpromazine (0.1 mg per ml in 5% dextrose, 1/2 normal saline) was initiated for a total dose of 0.5 to 1.1 mg per kg body weight at a rate of 60 ml per hour for 5 to 7 minutes. After administration of the acetylpromazine the animal was maintained on continuous intravenous infusion of 5% dextrose, 1/2 normal saline at a rate of 5 ml per hour throughout the remainder of the procedure. The skin was closed with a running 3-0 nylon suture.

The abdomen was then prepped with iodophor solution. A 4 to 5 cm transverse incision was made by a scalpel in the left upper quadrant approximately 1/3 of the distance between the xiphoid process and the umbilicus. The incision was continued down to the anterior rectus fascia. The left rectus muscle was bluntly dissected between clamps. The peritoneum was sharply entered and the stomach was identified and delivered into the incision. Double pursestring sutures of 4-0 dexon were placed in the center of the anterior surface of the stomach. An 8-French foley catheter was tunneled from a separate stab incision inferior to the first incision. The foley catheter was inserted via a small gastrostomy made by a scalpel. The inner pursestring suture was secured around the catheter, the stomach was pushed down slightly and the outer pursestring suture was secured. The 2 cc foley balloon was inflated with saline. The catheter was tested for patency with a wash of saline, using a foley catheter syringe. The anterior stomach was secured to the abdominal wall at two sites with 4-0 dexon. The abdomen was irrigated with 250 mg of cefazolin in 5 ml of sterile saline, and then closed anatomically in layers with 4-0 dexon. The skin was closed with a running 3-0 nylon suture. The intravenous silastic catheter and 20 gauge blunt needle were secured to the piglet's skin with silk. The gastrostomy tube was clamped with a rubber band. All external incisions were swabbed with iodophor solution.

Postsurgical Procedure

Following surgery, the piglets were transferred into the PNICU. Oxygen (95% O_2, 5% CO_2) was used as necessary to resuscitate the piglets. Piglet activity level and intravenous flow rate was monitored every 30 minutes. When adequate oral intake was accomplished, the intravenous infusion was discontinued. A luer-lock closure was placed on the external end of the intravenous catheter and the catheter was flushed with sterile heparinized saline. The intravenous catheter was flushed with heparinized saline once per day for the remainder of the experiment. The gastric catheter was flushed once per day with saline to prevent blockage.

Uptake Experiment

On Day Four of the experiment the piglets were fasted for four hours prior to the administration of a dose of 2 µCi of L-(CH_3)-^{14}C-carnitine (1 µCi per ml in sterile saline) at zero time. Each piglet received the tracer either intravenously (IV) through the internal jugular catheter or intragastrically (GT) through the gastrostomy tube. The dose was followed by sterile saline (0.5 ml for the IV, 1.0 ml for the GT) to clear the catheter tubing.

Sacrifice and Tissue Processing Procedure

At the appropriate time post-administration, the piglets were anesthetized by injection of thiamylal sodium (25 mg per kg body weight) into the internal jugular catheter. When anesthesia was acheived a ventral medial incision was made to expose the heart. The piglets were killed by exsanguition following venipuncture of the left ventriole of the heart. Whole blood was collected into EDTA prepared tubes and used in the determination of hematocrit and whole blood hemoglobin (Appendix A). The blood was then centrifuged at 3,000 x g for 15 minutes at 4 to 6°C to separate plasma and red blood cells. The red blood cells were washed twice with saline. Plasma and washed red blood cells were stored at -80°C.

The liver was removed from the body cavity and the gall bladder was dissected away from the tissue. The liver was then perfused with chilled saline to remove any blood. The heart ventricles and kidneys were trimmed of extraneous tissue and rinsed in chilled saline. The lungs, cerebrum, and cerebellum were trimmed and blotted gently to remove blood. The contents of the stomach, small and large intestine were collected. These tissues were then cut lengthwise, rinsed in chilled saline and blotted. The small intestine was divided into top, middle, and bottom small intestine segments of equal length. A skeletal muscle sample was collected from the gastrocnemius region of the hindlimb. Liver, heart, kidneys, lung, cerebrum, cerebellum, stomach, small intestine segments, and large intestine were blotted and weighed prior to storage at -80°C.

Assay Procedures

All samples were assayed for both radioactivity resulting from the tracer carnitine administration and for endogenous total carnitine levels. Tissue samples and gastrointestinal tract contents were hand homogenized in deionized water (1 g in 3 ml) using a Ten Broeck homogenizer (Fisher Scientific, Springfield, NJ). This homogenate was referred to as homogenate #1 (HOMO 1). Aliquots of HOMO 1 were used for the determination of levels of radioactivity. For samples such as gastrointestinal contents in which the carnitine level was expected to be low or undetectable, aliquots of HOMO 1 were used in the determination of total carnitine levels (Appendix A). For the other tissues, a second homogenate (HOMO 2) was made by diluting 1 ml of HOMO 1 in 2 ml of deionized water and rehomogenizing. For heart and skeletal muscle, in which the level of carnitine was expected to be higher, 0.5 ml of HOMO 1 was rehomogenized with 2.5 ml of deionized distilled water. Aliquots of this second homogentate were used for the determination of total carnitine and noncollagen protein levels (Appendix A).

All samples were solubilized prior to counting for radioactivity. Tissues, plasma, and bile were solubilized by aliquoting 0.4 ml of HOMO 1 or fluid sample into glass scintillation vials and adding 1 ml of Protosol (New England Nuclear, Boston, MA). Vials were then capped and incubated in a 55°C water bath for two hours (tissues) or one hour (fluid samples). If necessary, the samples were decolorized by the addition of 0.1 ml of 30% H_2O_2 and incubated for an additional 30 minutes. After cooling, 10 ml of Biofluor (New England Nuclear, Boston, MA) was added to the vials. Red blood cells were hemolyzed prior to solubilization by diluting with deionized water and refreezing. Samples of the thawed hemolysates were used for the carnitine assay and for the determination of red blood cell hemoglobin concentration (Appendix A). Aliquots of 0.2 ml of the hemolysate were solubilized by the addition of 0.5 ml of a Protosol-ethanol solution (1:2). Decolorization of the samples was achieved with the addition of 0.5 ml of 30% H_2O_2 and reincubation for 30 minutes. After cooling, the samples were combined with 15 ml of Biofluor and then neutralized by the addition of 0.5 ml of 0.5 N HCL. All samples remained in the dark for 5 to 10 days prior to counting in order to reduce chemiluminescence. The samples were counted in a liquid scintillation counter against a double label quench curve. Counts were considered acceptable when the random coincidence monitor values were 5% or below and when the channel 2 counting efficiency for isotope 2 (^{14}C) was 70% or greater.

Results and Discussion

The primary goal of this investigation was to develop methods and procedures which would allow use of the neonatal colostrum-deprived piglet as an animal model in investigations of neonatal carnitine metabolism and nutrition. An initial hypothesis that the domestic neonatal piglet would be a viable animal model for neonatal nutrition studies was made based on the similarities in physiology between human neonates and neonatal piglets. The finding that the patterns of carnitine accretion occurring during gestation in the piglet are similar to those observed in the human neonate further supports the use of the piglet as a model for human neonatal carnitine metabolism. A logical next step in the development of the piglet model, undertaken in the present investigation, was to attempt to deliver, maintain and use neonatal piglets in an initial study of tracer carnitine uptake in the neonatal animal. Colostrum-deprived piglets were chosen to mimic more closely the carnitine-free intake of the typical sick or preterm human neonate. Colostrum-deprivation also created a more metabolically fragile animal, again mimicing the neonatal human patient. This fragility made it necessary to develop clean room procedures similar to those used in neonatal intensive care units. In addition, it was necessary to maintain the neonatal animals in an environment of thermal neutrality (34 to 36°C). This again was similar to the situation in which the sick or preterm neonate would be maintained in an incubator. This temperature range allowed the piglets to maintain their body temperature without the metabolic stress of extensive thermogenesis (10).

Procedures for feeding the piglets were developed. A piglet formula and feeding protocol providing sufficient nutrition and fluids to maintain body weight and hydration status was the initial goal. This piglet formula was made from a commercial soy based infant formula (Appendix B). A comparison of published values of sow's milk composition and the infant formula suggested that the formula would be most comparable to sow's milk when prepared double-strength. Feeding frequency was modeled after the normal sow's pattern of many small volume feedings per day. The smaller volume feedings helped to prevent diarrhea or gut stasis from overfeeding, which would have inflicted metabolic stress on the piglets. As can be seen in Table 4-1, the piglets maintained their body weights over the four days of the experiment. A reduced growth rate similar to that observed in these piglets has been noted previously in early weaned piglets (92). Leece and King (92) referred to this slow rate of weight gain as the "post-weaning check." This lessened rate of growth may be advantageous in the use of the piglet model by making the piglet more comparable to the human infant in terms of growth rate. The mean hematocrit value (27.0 ± 5.2) was slightly lower than but still within the normal range for sow-fed four day old piglets (32.2 ± 3.1) (110). This would again indicate that the hydration status of the piglets was sufficiently maintained.

Table 4-1
Body Weights of Piglets (grams)

Day of Experiment	n	Mean ± SD	Range
Day One	14	1100 ± 202	(813-1420)
Day Four	14	1023 ± 234	(738-1380)

Note: Day One body weight is not significantly different from Day Four body weight ($p=0.0526$).

The surgical procedures, including anesthesia and post-surgical care, were modeled in part from human neonatal surgical techniques. The anesthesia regimen was based on both human neonatal techniques and techniques used in older swine (96) since no previously reported methods for neonatal swine were available. The decision to use labor induced vaginal delivery as opposed to caesarean derived piglets was based in part on a finding of less viable piglets from caesarean births, presumeably due to an effect of the sow's surgical anesthesia. The apparent inability of the neonatal piglet liver to metabolize the sow derived anesthesia (98) in combination with the anesthesia required for the surgical catheterization was overcome with the elimination of the caesarean section procedure. Another advantage of the labor induction procedure was that it provided more control over the time of piglet delivery. This was essential in coordinating the facilities and personnel required for piglet care in the PNICU and surgery. In addition, sows subjected to the labor induction procedure were successfully rotated back into the breeding herd.

The levels of total carnitine and protein determined in the tissues and blood of the piglets in this initial experiment will be discussed in combination with the results of the final experiment.

To summarize, this experiment was designed to answer the question of whether or not the neonatal colostrum-deprived piglet could be used as an animal model for investigating neonatal carnitine nutrition and metabolism. The neonatal piglet appears to be a good candidate for a neonatal model based on reports in the literature of similarities in physiology, size and metabolism. The results of the first experiment in this dissertation established the similarities in the gestational patterns of carnitine accretion between the human and piglet fetus. While these factors suggest that the piglet could be a good model for human neonatal carnitine metabolism, they do not establish the viability of the animals for actual investigations. The colostrum-deprived piglet is metabolically fragile, previously considered impossible to keep alive unless derived and maintained under strict sterile conditions (98,102). Human neonates, even preterm or sick neonates, are not maintained in germ-free conditions The piglets in this experiment survived well in clean room conditions very similar to those of the standard neonatal intensive care unit. Also like the human infants residing in a neonatal intensive care unit, these piglets received no exogenous carnitine or colostrum. The piglets were viable for the four days of the study, maintained their body weights, and withstood the venous and gastric surgical catheterization procedures. As will be discussed in the following chapter, tracer administered via these catheters was successfully detected in the piglet tissues. Thus, this experiment established that the neonatal colostrum-deprived

deprived piglet can be a viable animal model for investigating neonatal nutrition or metabolism. Further use of this animal model to investigate carnitine compartmentation and the influence of the route of carnitine administration in the neonate can now be pursued.

CHAPTER 5

AN INVESTIGATION OF TISSUE UPTAKE OF RADIOACTIVITY FROM TRACER CARNITINE ADMINISTERED INTRAVENOUSLY OR ENTERALLY TO NEONATAL COLOSTRUM-DEPRIVED PIGLETS

Introduction

This investigation was undertaken to further establish the use of the neonatal colostrum-deprived piglet model, to improve upon the techniques developed in the previous experiment and to investigate the patterns of tissue accumulation of radioactivity from intravenous and intragastric radiolabeled tracer carnitine administration. The intravenous and intragastric routes are the two potential routes of tracer administration to human neonates. An ideal comparision of the influence of the route of administration would allow comparision of the two routes in the same animal. This would eliminate the problem of animal to animal variation in metabolic state or carnitine status. Thus, in this experiment a double-label tracer system was developed, using both $^{14}C-(CH_3)$-L-carnitine and $^{3}H-(CH_3)$-L-carnitine as tracers. The site of the venous catheterization was changed from the internal jugular vein to the umbilical vein in order to decrease the surgical stress on the animals by eliminating the need for two incisions as a common abdominal incision was used for all catheters. This also resulted in improved catheter patency due to the larger vessel size.

Materials and Methods

Animals

All procedures involving piglets used in this study met with prior approval by the All University Committee on the Care and Use of Laboratory Animals. Piglets were the result of three-way crossbreeding (Duroc x Hampshire x Yorkshire) provided from the Research Breeding Herd through a collaborative research agreement with Dr. Fuller W. Bazer in the Department of Animal Science. Piglets were delivered on day 112 of gestation using the labor induction procedure described in the previous experiment. Following delivery the piglets were transported to the Piglet Neonatal Intensive Care Unit (PNICU) in the animal facility at the Food Science Building. Piglets were admitted into the PNICU as described previously.

Nutritional Support of Piglets

The piglets in this experiment received no oral feeds until formula could be taken following surgical recovery. Surgery was performed after delivery on Day One of the experiment. As in the last experiment, the piglets were maintained on continuous intravenous infusion of 5% dextrose, 1/2 normal saline until formula feeding was initiated. The piglet formula was altered by the addition of casein, mineral mix and corn oil to the carnitine free soy based infant formula base (Appendix B, Piglet Formula #2). The piglets were fed 10 ml of formula hourly on Day Two. On Day Three, the piglets were fed 25 and then 35 ml of formula every two hours. By Day Four the piglets were consuming 40 ml of formula every two hours. The dietary intake of the piglets was recorded at each feeding. The piglets were not fasted prior to tracer administration.

Catheter Preparation

Venous catheters were prepared from Silastic (Silastic Medical-Grade Tubing, Dow Corning Corporation) tubing (0.030 inches internal diameter, 0.065 external diameter) and Tygon (Norton Plastics and Synthetics) tubing (0.0625 inches internal diameter, 0.125 inches external diameter, 0.030 inches wall diameter). A 50 cm length of Silastic tubing was inserted into the Tygon tubing collar. The tip of the Silastic tubing was clamped with a hemostat and the Tygon collar was pulled 8 to 9 cm from the tip. The tip of the Silastic tubing was then cut to a length of 6 to 7 cm from the collar. The collar was secured to the Silastic tubing using a small amount of silicone rubber sealant (100% Clear Silicone Rubber Aquarium Sealant, Perfecto Manufacturing). The sealant was allowed to set and dry for two to three hours. The catheters were then individually wrapped and autoclaved.

Gastrostomy and urinary bladder catheters were prepared in a similar manner. The tubing used for these catheters was of larger diameter (Silastic: 0.062 inches internal diameter, 0.125 inches external diameter; Tygon: 0.094 inches internal diameter, 0.156 inches external diameter with a wall diameter of 0.030 inches). The Silastic tubing was approximately 32 cm in length with 2 to 3 cm between the Tygon collar and the tip. Several small openings were cut in the Silastic tubing between the collar and the tip. The collar was secured to the Silastic tubing and the catheters were wrapped and autoclaved in the same manner as the venous catheters.

Surgical Procedure

Surgery to insert intravenous, gastric, and urinary bladder catheters was performed on Day One (Day One equals day 112 gestation) of the experiment before feedings were initiated. Surgery was performed on two to four piglets per litter. Anesthesia was achieved using ketamine as described previously.

The site of venous catheterization was changed from the internal jugular vein used in the previous experiment to the umbilical vein. The abdomen was prepped with iodophor solution. An 8 to 9 cm ventral medial incision was made by a scalpel in the left external oblique muscle. The incision was initiated at the umbilicus and continued in the upper quadrant to the xiphoid process. The umbilical vein was identified and isolated by blunt dissection using a hemostat. A proximal loop and a distal loop (3-0 silk) were placed around the vessel. A small venotomy was made with venotomy scissors. A sterile 23 gauge butterfly needle was inserted into the venotomy to provide stability to the vein for catheter insertion. The custom made Silastic catheter (0.030 inches internal diameter, 0.065 inches external diameter) was inserted into the umbilical vein. The catheter tip was threaded upward into the vena cava. Patency was confirmed using a heparinized syringe and the catheter was then secured with two 3-0 silk ligatures, one placed distally and one placed proximally to the catheter collar. An intravenous drip of acetylpromazine was begun, followed by a continuous infusion of 5% dextrose, 1/2 normal saline as described previously.

The stomach was identified and delivered into the incision. The anterior surface of the stomach was identified and double pursestring sutures (4-0 dexon) were placed in the center. The custom made Silastic catheter (0.062 inches internal diameter, 0.125 inches external diameter) was inserted into the center of the pursestring sutures via a small gastrostomy made with a scalpel and enlarged by hemostats. The inner pursestring suture was secured around the catheter on the distal side of the catheter collar. The sutures were secured as described previously. A 16 gauge luer stub was inserted in the external tip of the catheter and capped with a luer lock adapter. The catheter was filled with sterile saline and tested for patency.

The abdominal incision was then continued into the lower left quadrant until the urinary bladder was identified. The urethra was identified and ligated with 3-0 silk. The urinary bladder was delivered into the incision. When necessary, the bladder was gently evacuated with a sterile 18 gauge needle and syringe. Double pursestring sutures of 4-0 dexon were placed in the bladder surface. The custom made Silastic catheter (0.062 inches internal diameter, 0.125 inches external diameter) was inserted into the center of the pursestring sutures through a small puncture made by a scalpel and enlarged with hemostats. The sutures were secured in the same manner as the gastrostomy sutures. A 16 gauge luer stub was inserted into the external tip of the catheter and capped with a luer lock adapter. The catheter was tested for patency with sterile saline. Urine was evacuated from the bladder via the catheter using a sterile needle and syringe every 30 minutes for the remainder of the experiment.

The abdomen was irrigated with 125 mg of cefazolin dissolved in 1 ml of sterile saline. The incision was then closed anatomically in layers with 4-0 dexon. The skin was closed with a 3-0 nylon running suture. The three catheters exited the incision at three separate sites near the umbilicus. The incision was swabbed with iodophor solution. The venous, gastric, and urinary bladder catheters were secured to the skin on the side and back of the piglet with cloth surgical tape. A bandage of sterile stretch terry cloth was placed around the piglet's midsection over the incision. The bandage opening through which the three catheters exited was placed at the piglet's spine and secured with tape. Postsurgical care was given as described previously.

Piglet Care Procedures

The piglets were weighed daily and received a daily intramuscular prophylactic dose of the broad spectrum antibiotic (cefazolin, 100 mg per kg body weight). The intravenous catheters were flushed with heparinized saline twice per day. The gastric catheters were flushed with saline to prevent blockage. The incision was inspected for edema or signs of infection and swabbed with iodophor solution once per day. A fresh sterile stretch terry cloth bandage was placed on the piglet after the incision was swabbed and was replaced as necessary when it became soiled. Urine was collected every 30 minutes and the volume collected was recorded. The dietary intake and activity level was recorded at each feeding. The piglet incubator boxes were swabbed with ethanol after each feeding to remove formula residue and prevent bacterial growth.

Uptake Experiment

Each piglet in this experiment received both an intravenous and an intragastric dose of radiolabeled tracer carnitine. The ^{14}C radiolabeled carnitine used in this experiment was L-methyl-^{14}C-carnitine hydrochloride (Amersham Corporation, Code CFA.668, Batch 8), prepared by demethylation and subsequent remethylation, with ^{14}C-methyl iodide, of unlabeled L-carnitine hydrochloride, purified by ion exchange chromatography and crystallization from ethanol. The specific activity of the undiluted radiolabeled carnitine is 58 mCi/mmol, 290 µCi/mg (molecular weight equal to 200 at this specific activity). The radiochemical concentration is 20 µCi/ml. The radiochemical purity of this material was assayed by the manufacturer using high performance liquid chromatography on microparticulate cation exchange resin using 0.2 M sodium citrate at constant ionic strength with a pH gradient of 2.2 to 11.5. The radiochemical purity determined by this method was 97 ± 0.4%. The ^{3}H radiolabeled carnitine was L-methyl-^{3}H-carnitine hydrochloride (Amersham Corporation, Code TRK.762, Batch 10), prepared by quaternization of L-4-dimethylamino-3-hydroxybutyric acid with ^{3}H-methyl iodide and purified by ion-exchange and high performance liquid chromatography. The specific activity of the undiluted radiolabeled carnitine was 72 Ci/mmol, 355 mCi/mg (molecular weight equal to 203 at this specific activity). The radiochemical concentration of this material is 1 mCi/ml. The radiochemical purity of this material was determined by the manufacturer as described above to be 97.5%. Aliquots of the L-methyl-^{14}C-carnitine and the L-methyl-^{3}H-carnitine were spotted on thin layer chromatographic plates (250 micron uniplate, Analtech Avicel) and developed using a propanol:NH_4OH:H_2O (17:1:3) solvent system. The location of the radioactivity of the plate scrapings corresponded to the location of the spot in the L-carnitine standard lane. At zero time, the piglets were administered an intragastric dose of 5 µCi ^{3}H-(CH_3)-L-carnitine (5 µCi ^{3}H/72.2 pmol carnitine/ml sterile saline) per kg body weight. This was followed by an intravenous dose of 2 µCi ^{14}C-(CH_3)-L-carnitine (2 µCi/34.5 nmol carnitine/0.5 ml sterile saline) per kg body weight. Both doses were followed by a wash of sterile saline (1.0 ml for the intragastric dose, 0.5 ml for the intravenous dose) to clear the catheter tubing. Feedings and urine collections continued following tracer administration. A preliminary experiment using this double label tracer carnitine administration system was performed with three piglets sacrificed at 15 minutes, three hours, and 24 hours post-administration. In the final experiment, six piglets were sacrificed at 15 minutes, 30 minutes, 1 hour, 3 hours, 5 hours, and 24 hours post-administration.

Sacrifice and Tissue Processing

The piglets were anesthetized and killed as described in the previous experiment. Tissues were collected and processed as before. In addition, the spleen, adrenals, and a bone marrow sample were collected. The bone marrow sample was collected by aspirating 0.5 ml of saline from the femur. The spleen and adrenals were trimmed of extraneous tissue, rinsed in chilled saline, blotted and weighed. All samples were frozen at -80°C until assay.

Statistical Analyses

The statistical analyses of data were performed using the RS/1 Integrated Data Analysis System for the Digital Professional 350 (Digital Equipment Corporation, Maynard, MA). Sample populations were subjected to the Wilk-Shapiro test of normality. Normal populations were tested for equal or unequal variances with an F-test for variance ratio. A T-test for either equal or unequal variances was then performed. In the case of non-normal populations the Ansari-Bradley test for equal dispersions was used, followed by the Mann-Whitney test for unpaired samples with equal dispersions (107).

Results and Discussion

The body weights of the two groups of double label tracer piglets are shown in Table 5-1. While the birth and Day Four body weights did not differ in the three preliminary piglets, a paired comparison of the weights of the final group of piglets revealed a slight increase in body weight between birth and Day Four. Thus these piglets were also able to maintain their body weights throughout the four days of the study.

The carnitine levels in the blood and tissues of the piglets from the previous experiment and the two groups of piglets in the present study were compared. The carnitine values of the plasma and red blood cells are shown in Table 5-2. The three groups of piglets did not differ in blood levels of carnitine although the three preliminary piglets tended to have lower values. Bile carnitine levels (Table 5-3) did not differ between the piglets. As described, a bone marrow hemolysate was collected from the piglets used in the present study. These values, shown in Table 5-4, appear similar, although a statistical comparision was not possible due to the small sample size of the preliminary piglets. The parameters measured in the cardiac and skeletal muscle tissues of the three groups of piglets are shown in Table 5-5 and 5-6. In both of these tissues the total carnitine levels expressed per mg noncollagen protein were higher in the first group of piglets than in the piglets of the third

Table 5-1
Birth and Day Four Body Weights of Piglets from Experiment 3

Preliminary Group

	Birth	Day Four
Body Weight (g)	961 ± 98 (3)	983 ± 88 (3)

Note: Paired comparision reveals no significant difference between value at Birth and Day Four, P= 0.3841.

Final Group

	Birth	Day Four
Body Weight (g)	1553 ± 216 (6)	1579 ± 209 (6)

Note: Paired comparision indicates that value at Day Four is significantly higher then value at Birth, P= 0.0085

Table 5-2
Carnitine Values of Plasma and Red Blood Cells of Piglets from Experiment 2 and Experiment 3

	Experiment 2	Experiment 3 (Preliminary)	Experiment 3
Plasma: (nmol/ml)	33.2 ± 17.3^{A} (14)	12.7 ± 6.2^{A} (3)	28.3 ± 16.9^{A} (6)
Red Blood Cells: (nmol/mg hemoglobin)	$0.05 \pm .020^{A}$ (14)	$0.026 \pm .005^{A}$ (3)	$0.05 \pm .037^{A}$ (6)

Note Values within a row with different superscripts are significantly different.

Table 5-3
Carnitine Levels of Bile from Piglets of Experiment 2 and 3

	Experiment 2	Experiment 3 (Preliminary)	Experiment 3
Total Carnitine: (nmol/ml)	13.6 ± 8^{A} (13)	7.8 ± 4.2^{A} (3)	21.5 ± 11.7^{A} (6)

Note: Values within a row with different superscripts are significantly different.

Table 5-4
Carnitine Levels of Bone Marrow from Piglets of Experiment 3

	Experiment 3 (Preliminary)	Experiment 3
Total Carnitine (nmol/mg hemoglobin)	2.27 ± .20* (2)	2.10 ± .91 (6)

Note: *Statistical comparison not possible due to small sample size.

Table 5-5

Heart Tissue Parameters of Piglets from Experiment 2 and 3

	Experiment 2	Experiment 3 (Preliminary)	Experiment 3
Total Carnitine: (nmol/mg protein)	12.19 ± 2.56^{A} (14)	6.66 ± 1.27^{B} (3)	8.01 ± 1.11^{B} (6)
Total Carnitine: (nmol/g)	1090 ± 220^{A} (14)	957 ± 201^{A} (3)	1020 ± 312^{A} (6)
Total Carnitine: (nmol/organ/kg)	5550 ± 1440^{A} (13)	5420 ± 327^{A} (3)	5210 ± 1870^{A} (6)
Relative Organ Size (%):	$0.52 \pm .08^{A}$ (13)	$0.58 \pm .08^{A}$ (3)	$0.51 \pm .04^{A}$ (6)
Noncollagen Protein: (mg/g)	91 ± 18^{A} (14)	143 ± 3^{B} (3)	127 ± 31^{B} (6)

Note: Values within a row with different superscripts are significantly different from each other. Numbers in parentheses indicate number of samples.

Table 5-6
Muscle Parameters of Piglets from Experiment 2 and 3

	Experiment 2	Experiment 3 (Preliminary)	Experiment 3
Total Carnitine: (nmol/mg protein)	11.87 ± 3.70^{A} (9)	$6.79 \pm .38^{B}$ (3)	8.82 ± 3.20 (6)
Total Carnitine: (nmol/g)	1060 ± 220^{A} (9)	1070 ± 115^{A} (3)	1299 ± 346^{A} (6)
Total Carnitine: (nmol/organ/kg)	268000 ± 55500^{A} (9)	270000 ± 29000^{A} (3)	327000 ± 87200^{A} (6)
Noncollagen Protein: (mg/g)	90 ± 20^{A} (9)	157 ± 11^{B} (3)	152 ± 22^{B} (6)

Note: Values within a row with different superscripts are significantly different from each other. Numbers in parentheses indicate number of samples.

experiment. This difference is likely due to the lower protein levels measured in both heart and skeletal muscle in the first group of piglets, especially since the total carnitine levels expressed per g wet weight and per organ did not differ. The lower tissue protein levels of the first group of piglets may have resulted from the piglets being in a cachectic condition. This could have been the result of metabolic stress brought about by the surgery or the composition of the first piglet diet formula. The second group of piglets benefited from both improved surgical techniques and improved diet formulation, including the addition of casein to the formula (Appendix B). The carnitine levels of the cardiac and skeletal muscle tissues, expressed per mg noncollagen protein and per g wet weight are similar for each group of piglets.

The liver total carnitine levels, relative organ sizes and noncollagen protein levels are shown in Table 5-7. The primary differences seem to be due to a smaller relative liver size of the three preliminary piglets of the third experiment. As was observed for the cardiac and skeletal muscle, the noncollagen protein levels of the livers of the first group of piglets were low. This again may have resulted from a cachectic condition. In general, the liver carnitine levels were variable. This could reflect carnitine mobilization from other tissues and possibly carnitine

Table 5-7
Liver Parameters of Piglets from Experiment 2 and 3

	Experiment 2	Experiment 3 (Preliminary)	Experiment 3
Total Carnitine: (nmol/mg protein)	$2.59 \pm .88^{A}$ (13)	2.48 ± 1.40^{A} (3)	$3.09 \pm .78^{A}$ (6)
Total Carnitine: (nmol/g)	258 ± 94^{A} (13)	312 ± 153^{A} (3)	360 ± 124^{A} (6)
Total Carnitine: (nmol/organ/kg)	6400 ± 3350 (14)	6740 ± 4020^{A} (3)	9980 ± 4110^{B} (6)
Relative Organ Size (%):	$2.73 \pm .37^{A}$ (14)	$2.08 \pm .25^{B}$ (3)	$2.75 \pm .31^{A}$ (6)
Noncollagen Protein (mg/g):	100 ± 19^{A} (13)	129 ± 9^{B} (3)	115 ± 10 (6)

Note: Values within a row with different superscripts are significantly different. Numbers in parentheses indicate number of samples.

synthesis, although the carnitine biosynthetic capability of these piglets is not known.

The kidney parameters are displayed in Table 5-8. Like the liver values, the total carnitine levels of this tissue varied between the groups of piglets. Total carnitine levels expressed per mg noncollagen protein and per g wet weight were highest in the kidneys of the first group of piglets. These levels were lowest in the final group of piglets. Although not statistically significant, the kidneys of the final group of piglets tended to be larger than the other piglets. A larger organ size might explain the lower levels of total carnitine per g wet weight or per mg noncollagen protein. As can be seen in Table 5-8, the total carnitine per organ, normalized to a kg body weight, did not differ significantly between the three groups of piglets. Variations in carnitine levels in the kidneys could reflect carnitine synthesis or renal filtration from the plasma.

The values for lung tissue from the three groups of piglets are shown in Table 5-9. While there were no significant differences in relative lung size or noncollagen level between the three groups of piglets, the carnitine levels of the first group of piglets were consistently higher than the other two groups. The carnitine and protein values measured in the piglet brains are shown in Table 5-10 and 5-11. Brain carnitine levels tended to be low relative to the other tissues.

Table 5-8
Kidney Parameters of Piglets from Experiment 2 and 3

	Experiment 2	Experiment 3 (Preliminary)	Experiment 3
Total Carnitine (nmol/mg protein):	4.75 ± 2.61^{A} (14)	$1.48 \pm .34^{B}$ (3)	$3.23 \pm .72^{C}$ (8)
Total Carnitine (nmol/g):	370 ± 180^{A} (14)	123 ± 18^{B} (3)	228 ± 60^{C} (8)
Total Carnitine (nmol/organ/kg):	2840 ± 1880^{A} (13)	877 ± 453^{A} (3)	2080 ± 923^{A} (8)
Relative Organ Size (%):	$0.72 \pm .15^{A}$ (13)	$0.70 \pm .31^{A}$ (3)	$0.89 \pm .21^{A}$ (8)
Noncollagen Protein (mg/g):	82 ± 17^{A} (14)	88 ± 11^{A} (3)	71 ± 8^{A} (6)

Note: Values within a row with different superscripts are significantly different. Numbers in parentheses indicate number of samples.

Table 5-9
Lung Parameters of Piglets from Experiment 2 and 3

	Experiment 2	Experiment 3 (Preliminary)	Experiment 3
Total Carnitine (nmol/mg protein):	$1.80 \pm .60^{A}$ (14)	$0.53 \pm .21^{B}$ (3)	$1.10 \pm .33^{C}$ (6)
Total Carnitine (nmol/g)	132 ± 39^{A} (14)	37 ± 20^{B} (3)	76 ± 38^{B} (6)
Total Carnitine (nmol/organ/kg):	2580 ± 395^{A} (14)	573 ± 282^{B} (3)	1200 ± 314^{C} (6)
Relative Organ Size (%):	$1.91 \pm .32^{A}$ (14)	$1.56 \pm .06^{A}$ (3)	$1.80 \pm .55^{A}$ (6)
Noncollagen Protein (nmol/g):	75 ± 8^{A} (14)	68 ± 9^{A} (3)	67 ± 20^{A} (6)

Note: Values within a row with different superscripts are significantly different. Numbers in parentheses indicate number of samples.

Table 5-10
Cerebrum Parameters of Piglets from Experiment 2 and 3

	Experiment 2	Experiment 3 (Preliminary)	Experiment 3
Total Carnitine (nmol/mg protein):	$0.69 \pm .22^{A}$ (14)	$0.54 \pm .15^{A}$ (3)	$0.96 \pm .36^{A}$ (6)
Total Carnitine (nmol/g)·	52 ± 16^{A} (14)	36 ± 6^{A} (3)	74 ± 35^{A} (6)
Total Carnitine (nmol/organ/kg):	1460 ± 729^{A} (13)	634 ± 133^{B} (3)	1390 ± 776 (6)
Relative Organ Size (%):	2.86 ± 1.39^{A} (13)	$2.19 \pm .16$ (3)	$1.62 \pm .26^{B}$ (6)
Noncollagen Protein (mg/g):	75 ± 10^{A} (14)	72 ± 6^{A} (3)	75 ± 7^{A} (6)

Note: Values within a row with different superscripts are significantly different. Numbers in parentheses indicate the number of samples.

Table 5-11
Cerebellum Parameters of Piglets from Experiment 2 and 3

	Experiment 2	Experiment 3 (Preliminary)	Experiment 3
Total Carnitine (nmol/mg protein):	0.73 ± .20^A (14)	0.82 ± .09 (3)	0.98 ± .26^B (6)
Total Carnitine (nmol/g):	58 ± 17^A (14)	62 ± 6 (3)	81 ± 24^B (6)
Total Carnitine (nmol/organ/kg):	409 ± 159^A (13)	372 ± 122^A (3)	258 ± 111^A (6)
Relative Organ Size (%):	0.67 ± .18^A (13)	0.60 ± .17 (3)	0.31 ± .04^B (6)
Noncollagen Protein (mg/g):	78 ± 7^A (14)	75 ± 2^A (3)	83 ± 6^A (6)

Note: Values within a row with different superscripts are significantly different. Numbers in parentheses indicate number of samples.

The total carnitine and protein levels in the tissues and contents of the gastrointestinal tract are shown in Tables 5-12 to 5-19. Total carnitine levels expressed per mg noncollagen protein were highest in the three preliminary piglets, however, this could be explained by the tendency of their stomach tissue to be lower in noncollagen protein. Values measured in stomach contents (Table 5-13) were variable. The lower protein levels in the contents of the first group of piglets could be explained by the fact that these piglets were fasted during the uptake experiment. The second two groups of piglets continued feedings after tracer administration. While piglet feedings continued on schedule, the times of sacrifice varied from piglet to piglet, resulting in differing amounts of contents. The total carnitine concentrations of the top and middle sections of small intestine (Tables 5-14 and 5-15) were highest in the first group of piglets. The total carnitine levels tended to be higher in the bottom small intestine section of the first group of piglets also, although this was not statistically significant (Table 5-16). Like the gastric contents, the small intestine contents total carnitine and noncollagen protein levels were variable (Table 5-17). Large intestine tissue total carnitine levels (Table 5-18) followed a pattern similar to that observed in the small intestine. Again, the total carnitine levels of the first group of piglets were higher than the levels in the other groups of piglets. The

Table 5-12
Stomach Parameters of Piglets from Experiment 2 and 3

	Experiment 2	Experiment 3 (Preliminary)	Experiment 3
Total Carnitine (nmol/mg protein):	$1.45 \pm .46^{A}$ (14)	$0.78 \pm .22^{B}$ (3)	$1.18 \pm .15^{A}$ (6)
Total Carnitine (nmol/g):	120 ± 35^{A} (14)	80 ± 28^{A} (3)	108 ± 8^{A} (6)
Total Carnitine (nmol/organ/kg):	673 ± 226^{A} (13)	384 ± 102 (3)	451 ± 68^{B} (6)
Relative Organ Size (%):	$0.55 \pm .07^{A}$ (13)	$0.49 \pm .06^{A}$ (3)	$0.41 \pm .04^{B}$ (6)
Noncollagen Protein (mg/g).	84 ± 11^{A} (14)	101 ± 8^{B} (3)	92 ± 8 (6)

Note: Values within a row with different superscripts are significantly different. Numbers in parentheses indicate numbers of samples.

Table 5-13
Stomach Content Parameters of Piglets from Experiment 2 and 3

	Experiment 2	Experiment 3 (Preliminary)	Experiment 3
Total Carnitine (nmol/mg protein):	$0.37 \pm .39^{A}$ (14)	$0.01 \pm .003^{B}$ (3)	$0.03 \pm .04^{B}$ (6)
Total Carnitine (nmol/g):	2.87 ± 4.10^{A} (14)	$0.26 \pm .007^{A}$ (3)	2 ± 3^{A} (6)
Total Carnitine (nmol/pool/kg):	*	12 ± 4^{A} (3)	67 ± 127^{A} (6)
Relative Organ Size (%):	*	4.39 ± 1.33^{A} (3)	$3.61 \pm .76^{A}$ (6)
Noncollagen Protein (mg/g):	9 ± 6^{A} (14)	24 ± 5^{B} (3)	73 ± 45^{C} (6)

Note: Values within a row with different superscripts are significantly different. Numbers in parentheses indicate number of samples. *Not determined.

Table 5-14
Parameters of Top Small Intestine of Piglets
from Experiment 2 and 3

	Experiment 2	Experiment 3 (Preliminary)	Experiment 3
Total Carnitine (nmol/mg protein):	1.88 ± .90^{A} (14)	0.62 ± .30^{B} (3)	0.75 ± .09^{B} (6)
Total Carnitine (nmol/g):	164 ± 96^{A} (14)	65 ± 26 (3)	80 ± 16^{B} (6)
Total Carnitine (nmol/organ/kg):	1470 ± 664^{A} (14)	683 ± 267 (3)	742 ± 201^{B} (6)
Relative Organ Size (%):	0.86 ± .31^{A} (14)	1.07 ± .16^{1A} (3)	0.92 ± .06^{A} (6)
Noncollagen Protein (mg/g):	99 ± 15^{A} (14)	109 ± 14^{A} (3)	106 ± 10^{A} (6)

Note: Values within a row with different superscripts are significantly different. Numbers in parentheses indicate number of samples.

Table 5-15
Parameters of Middle Small Intestine of Piglets from Experiment 2 and 3

	Experiment 2	Experiment 3 (Preliminary)	Experiment 3
Total Carnitine (nmol/mg protein):	$1.69 \pm .57^{A}$ (13)	$0.65 \pm .21^{B}$ (3)	$0.86 \pm .13^{B}$ (6)
Total Carnitine (nmol/g):	172 ± 52^{A} (13)	69 ± 18^{B} (3)	88 ± 18^{B} (6)
Total Carnitine (nmol/organ/kg):	1360 ± 529^{A} (13)	879 ± 110 (3)	779 ± 272^{B} (6)
Relative Organ Size (%):	$0.79 \pm .25^{A}$ (13)	$1.34 \pm .22^{B}$ (3)	$0.87 \pm .18^{A}$ (6)
Noncollagen Protein (mg/g):	105 ± 25^{A} (13)	106 ± 5^{A} (3)	101 ± 10^{A} (6)

Note: Values within a row with different superscripts are significantly different. Numbers in parentheses indicate number of samples

Table 5-16
Parameters of Bottom Small Intestine of Piglets from Experiment 2 and 3

	Experiment 2	Experiment 3 (Preliminary)	Experiment 3
Total Carnitine (nmol/mg protein):	1.42 ± .72^{A} (14)	0.60 ± .21^{A} (3)	1.13 ± .37^{A} (6)
Total Carnitine (nmol/g):	140 ± 75^{A} (14)	77 ± 24^{A} (3)	112 ± 40^{A} (6)
Total Carnitine (nmol/organ/kg).	1240 ± 693^{A} (14)	903 ± 207^{A} (3)	1070 ± 482^{A} (6)
Relative Organ Size (%):	0.91 ± .29^{A} (14)	1.20 ± .25^{A} (3)	0.95 ± .18^{A} (6)
Noncollagen Protein (mg/g):	100 ± 17^{A} (14)	96 ± 5^{A} (3)	99 ± 7^{A} (6)

Note: Values within a row with different superscripts are significantly different. Numbers in parentheses indicate number of samples.

Table 5-17
Parameters of Small Intestine Contents of Piglets from Experiment 2 and 3

	Experiment 2	Experiment 3 (Preliminary)	Experiment 3
Total Carnitine (nmol/mg protein):	$1.19 \pm .85^{A}$ (14)	$1.60 \pm .69$ (3)	$2.16 \pm .97^{B}$ (6)
Total Carnitine (nmolg):	13 ± 11^{A} (14)	23 ± 9 (3)	45 ± 25^{B} (6)
Total Carnitine (nmol/organ/kg):	*	216 ± 193^{A} (3)	766 ± 380^{A} (6)
Relative Organ Size (%):	*	$1.67 \pm .16^{A}$ (3)	$1.77 \pm .34^{A}$ (6)
Noncollagen Protein (mg/g):	10 ± 6^{A} (14)	15 ± 3 (3)	22 ± 9^{B} (6)

Note: Values within a row with different superscripts are significantly different. Numbers in parentheses indicate number of samples. *Not Determined.

Table 5-18
Parameters of Large Intestine of Piglets
from Experiment 2 and 3

	Experiment 2	Experiment 3 (Preliminary)	Experiment 3
Total Carnitine (nmol/mg protein):	1.64 ± .34^{A} (13)	0.70 ± .37^{B} (3)	1.16 ± 29^{B} (6)
Total Carnitine (nmol/g):	131 ± 38^{A} (13)	87 ± 35 (3)	92 ± 30^{B} (6)
Total Carnitine (nmol/organ/kg):	1380 ± 832^{A} (13)	463 ± 168 (3)	593 ± 224^{B} (6)
Relative Organ Size (%):	0.99 ± .47^{A} (14)	0.54 ± .06^{B} (3)	.64 ± .04^{C} (6)
Noncollagen Protein (mg/g):	82 ± 15^{A} (14)	129 ± 15^{B} (3)	78 ± 8^{C} (6)

Note: Values within a row with different superscripts are significantly different. Numbers in parentheses indicate number of samples.

Table 5-19
Parameters of Large Intestine Contents of Piglets from Experiment 2 and 3

	Experiment 2	Experiment 3 (Preliminary)	Experiment 3
Total Carnitine (nmol/mg protein):	2.58 ± 2.17^A (14)	$1.42 \pm .35^A$ (3)	2.65 ± 1.61^A (6)
Total Carnitine (nmol/g):	8 ± 4^A (14)	19 ± 9^B (3)	83 ± 35^C (6)
Total Carnitine (nmol/pool/kg)	*	28 ± 22^A (3)	432 ± 342^B (6)
Relative Organ Size (%):	*	$0.39 \pm .15^A$ (3)	$0.51 \pm .26^A$ (6)
Noncollagen Protein (mg/g):	5 ± 5^A (14)	13 ± 4^B (3)	41 ± 27^C (6)

Note: Values within a row with different superscripts are significantly different. Numbers in parentheses indicate number of samples. *Not Determined.

total carnitine and protein levels measured in the large intestine contents (Table 5-19) varied greatly between the three groups of piglets.

The total carnitine levels and protein concentrations of the spleen and adrenals of the piglets from the present experiment are shown in Table 5-20 and 5-21. These tissues were not sampled from the piglets of the previous experiment. In both of these tissues the only significant differences between the two groups of piglets were in the the total carnitine levels per organ, normalized to a kg body weight In both cases the values for the second group of piglets were higher. The total urine values for urine collected over the four days of the study are shown in Table 5-22.

The investigation of the tissue accumulation of radioactivity from tracer carnitine administration in this study is unique for several reasons. First, this is the first experiment in which in vivo tracer carnitine administration has been investigated in a neonatal model. Secondly, this study represents a first attempt at investigation of intragastric tracer carnitine administration in a whole animal. This study also represents the first use of the piglet model in an investigation of this type. The tissue uptake of intravenously administered radiolabeled carnitine has previously been investigated in both adult dogs and adult rats (75,76,79). The patterns of radioactivity

Table 5-20
Parameters of Spleen Tissue of Piglets from Experiment 3

	Experiment 3 (Preliminary)	Experiment 3
Total Carnitine (nmol/mg protein)	1.34 ± .43^{A} (3)	1.19 ± .25^{A} (6)
Total Carnitine (nmol/g):	118 ± 45^{A} (3)	138 ± 32^{A} (6)
Total Carnitine (nmol/organ/kg)	89 ± 40^{A} (3)	141 ± 18^{B} (6)
Relative Organ Size (%):	0.07 ± .02^{A} (3)	0.11 ± .03^{A} (6)
Noncollagen Protein (mg/g):	101 ± 61^{A} (3)	115 ± 7^{A} (6)

Note: Values within a row with different superscripts are significantly different. Numbers in parentheses indicate number of samples.

Table 5-21
Parameters of Adrenals of Piglets from Experiment 3

	Experiment 3 (Preliminary)	Experiment 3
Total Carnitine (nmol/mg protein)	$0.42 \pm .13^{A}$ (3)	$0.56 \pm .21^{A}$ (6)
Total Carnitine (nmol/g):	40 ± 8^{A} (3)	96 ± 47^{A} (6)
Total Carnitine (nmol/organ/kg)	$7 \pm .5^{A}$ (3)	18 ± 9^{B} (6)
Relative Organ Size (%):	$0.02 \pm .003^{A}$ (3)	$0.02 \pm .003^{A}$ (6)
Noncollagen Protein (mg/g):	97 ± 23^{A} (3)	162 ± 101^{A} (6)

Note: Values within a row with different superscripts are significantly different. Numbers in parentheses indicate number of samples.

Table 5-22
Urine Parameters of Piglets from Experiment 3
(Values represent totals of 4 days of collections)

	Total Volume (ml)	Total Carnitine (nmol)	Total Creatinine (mg)
Piglet #1:	164.9	14,400	81.16
Piglet #2:	103.0	2960	69.30
Piglet #3:	243.7	4680	263.27
Piglet #4:	160.6	2810	54.86
Piglet #5:	159.1	2650	52.85
Piglet #6:	122.7	2310	78.61

Note: Values represent totals for urine collected during the four days of the study following bladder catheterization procedure.

observed in the piglet tissues (linearity is not implied by the graphic display of these patterns) will be discussed and contrasted with the patterns reported in the other species, although comparisions are difficult due to the differences in species, route of administration and age of the animals.

Brooks and McIntosh (79) base their analysis of the fate of tritium labeled carnitine in adult male rats on two assumptions. These assumptions are that blood plasma is the reservoir of labeled carnitine and that plasma is the only path by which carnitine may travel from tissue to tissue. In the present study, the accumulation of radioactivity in the red blood cells from either route of administration was negligible in the piglets. Although lymph was not measured in this study, other investigators (69) have reported negligible uptake of tracer in this system. Thus, in this investigation it will be assumed that plasma does represent the primary pathway of tracer carnitine between tissues. Figures 5-1 and 5-2 display the radiospecific activity of plasma resulting from both routes of administration in the two groups of double-labeled piglets. In both sets of piglets, the radioactivity in the plasma resulting from the intravenous administration decreases with time following administration. The pattern of radioactivity in the plasma resulting from intragastric administration is more variable. This may be indicative of slower release of the tracer from the gastrointestinal tract.

Figure 5-1: Plasma radiospecific activity versus time from intravenous (IV) and intragastric (GT) administration of radiolabeled carnitine in the preliminary group of piglets.

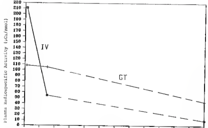
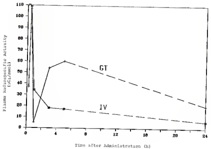

Plasma Radiospecific Activity (µCi/mmol)
220
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
IV
GT
0
4
8
12
16
20
24
Time after Administration (h)

Figure 5-2: Plasma radiospecific activity versus time from intravenous (IV) and intragastric (GT) administration of radiolabeled carnitine in the final group of piglets.


Plasma Radiospecific Activity
(μCi/mmol)
110
100
90
80
70
60
50
40
30
20
10
0
GT
IV
0
4
8
12
16
20
24
Time after Administration (h)

The pattern of radioactivity from intragastrically administered tracer observed in the tissues of the gastrointestinal tract is shown in Figure 5-3. The highest counts were found in the top intestine of the final group of piglets. This may reflect uptake and possibly retention of the tracer from the intestinal lumen. Other workers (69,70,71) have reported that the highest uptake and concentration of carnitine (nonlabeled) from an intraluminal dose occurs in the proximal intestine. The amount of radioactivity resulting from the intragastrically administered tracer found in the gastrointestinal contents (data not shown) decreased with time postadministration. Gastric contents contained 20 to 49% of the intragastrically administered label between 15 minutes and one hour postadministration. By 24 hours postadministration this % had decreased to less than 1%. Similarly, small intestine contents decreased in amount of radioactivity to 1% at 24 hours postadministration. Large intestine contents levels of radioactivity were consistently low, never rising above 1% of the administered dose. The amount of radioactivity from the intravenous dose found in the gastrointestinal tissues was consistently low (Figure 5-4).

Other investigators (71) have reported uptake of radiolabeled carnitine into the bile. Gudjonsson et al. (71) detected small amounts of radioactivity from an intravenous and an intraluminal injection of tracer carnitine in the bile

Figure 5-3: Radioactivity versus time from intragastrically administered radiolabeled carnitine in stomach (STO), top small intestint (TI), middle small intestine (MI), bottom small intestine (BI), and large intestine (LI).

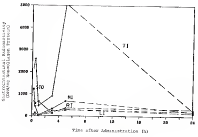
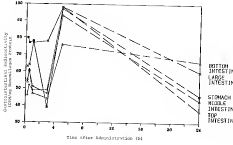

Gastrointestinal Radioactivity
(DPM/mg Noncollagen Protein)
5000
4000
3000
2000
1000
0
0
4
8
12
16
20
24
Time after Administration (h)
TI
STD
MI
BI
LI

Figure 5-4: Radioactivity versus time from intravenously administered radiolabeled carnitine in gastrointestinal tissue.


Gastrointestinal Radioactivity
(DPM/mg Noncollagen Protein)
100
90
80
70
60
50
40
30
0
4
8
12
16
20
24
Time after Administration (h)
BOTTOM INTESTINE
LARGE INTESTINE
STOMACH
MIDDLE INTESTINE
TOP INTESTINE

of cannulated adult male rats. Figures 5-5 and 5-6 show the patterns of accumulation of radioactivity in bile (dpm/ml) and liver (dpm/mg noncollagen protein) from the intravenous and intragastric doses of tracer carnitine in the piglets. While the amount of radioactivity in the bile from either dose never exceeds approximately 400 dpm/ml, this level is achieved much earlier with the intravenously administered dose (Figure 5-5). The slower appearance of label in the bile from the intragastric dose (Figure 5-6) again may reflect the release and subsequent uptake by the liver of label from the gastrointestinal tract.

One difficulty common to investigations of tracer carnitine administration is the question of accounting for the total dose of labeled carnitine administered. Cederblad and Lindstedt (78) reported an unexplained discrepancy between the calculated elimination of carnitine and the amount actually found in the urine. The percentages of the total doses of ^{14}C and ^{3}H recovered in the piglet tissues are summarized in Table 5-23. The skeletal muscle accounted for the majority of detectable radioactivity from both routes of administration in the piglets (Figures 5-7 to 5-10), yet this percentage never exceeded 40% of the administered dose. Yue and Fritz (75) was able to account for approximately 97% of intravenously administered tritium-labeled D,L-carnitine in one dog by assuming that the skeletal muscle mass in mongrel dogs represented 45% of their body weight. Rebouche and

Figure 5-5: Radioactivity in bile [DPM (x 10^3)/ml] and liver [DPM (x 10^3)/mg noncollagen protein] versus time from intravenously administered radiolabeled carnitine.

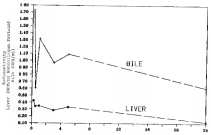

Radioactivity
Liver (DPM/mg Noncollagen Protein)
Bile (DPM/ml)
1.80
1.70
1.60
1.50
1.40
1.30
1.20
1.10
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0
4
8
12
16
20
24
Time after Administration (h)
BILE
LIVER

Figure 5-6: Radioactivity in bile [DPM $(\times 10^3)$/ml] and liver [DPM $(\times 10^3)$/mg noncollagen protein] versus time from intragastrically administered radiolabeled carnitine.

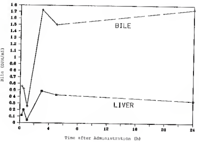

Radioactivity
Liver (DPM/mg Noncollagen Protein)
Bile (DPM/ml)
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
BILE
LIVER
0
4
8
12
16
20
24
Time after Administration (h)

Table 5-23
Summary of the Percentages of Total Doses of ^{14}C and ^{3}H Recovered in Piglet Tissues and Excreta

	Time of Collection (Post-Administration)	Total % ^{14}C	Total % ^{3}H
Piglet #1:	15 minutes	34	42
Piglet #2:	30 minutes	33	34
Piglet #3:	1 hour	41	55
Piglet #4:	3 hours	52	46
Piglet #5	5 hours	46	51
Piglet #6:	24 hours	47	44

Figure 5-7: Percent of total intravenously administered radioactivity versus time in heart, muscle, liver, and kidney of the preliminary piglets.

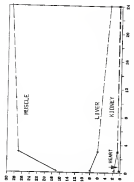

Percent of Total Dose (%)
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
Time after Administration (h)
0
4
8
12
16
20
24
MUSCLE
LIVER
KIDNEY
HEART

Figure 5-8: Percent of total intragastrically administered radioactivity versus time in heart, muscle, liver and kidney of the preliminary piglets.

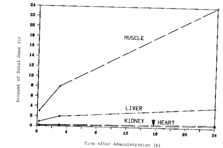

MUSCLE
LIVER
KIDNEY
HEART
Percent of Total Dose (%)
0
2
4
6
8
10
12
14
16
18
20
22
24
Time after Administration (h)
0
4
8
12
16
20
24

Figure 5-9: Percent of total intravenously administered radioactivity versus time in heart, muscle, liver and kidney of the final group of piglets.

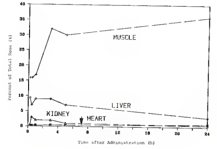
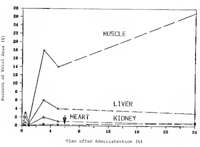

Percent of Total Dose (%)
40
35
30
25
20
15
10
5
0
MUSCLE
LIVER
KIDNEY
HEART
0
4
8
12
16
20
24
Time after Administration (h)

Figure 5-10: Percent of total intragastrically administered radioactivity versus time in heart, muscle, liver and kidney of the final group of piglets.


28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
Percent of Total Dose (%)
0
4
8
12
16
20
24
Time after Administration (h)
MUSCLE
LIVER
HEART
KIDNEY

Engel (76) estimated the muscle mass of mongrel dogs at 35% of body weight, but did not account for the total dose administered. The muscle mass of the piglets was estimated at 25.2% of their body weights, based on values reported in the literature (112). Liver and then kidney contained the next highest percentage of the administered radioactivity in the piglets. The percent of the total administered radioactivity from either route of administration never exceeded 6% in the plasma of the piglets. Similarly, the highest total urinary excretion of radioactivity (data not shown), that of the 24 hour piglet, only accounted for 6.4% of the intragastric dose and 2.5% of the intravenous dose. Clearly, the destination of the remainder of the radioactivity resulting from the radiolabeled carnitine remains unexplained. While the possibility of further metabolism or degradation of carnitine has not been investigated in the piglet, no further metabolism was found in rat tissues (78). A carnitine decarboxylation product, β-methylcholine, has been detected in urine of starved, diabetic or high-fat fed rats (13). Further efforts to identify this reaction in rat tissues have been unsuccessful (75,78). Yue and Fritz (75) reported an unidentified phospholipid fraction containing radioactivity in the skeletal muscle from an intravenously administered dose of carnitine in dogs. This fraction accounted for less than 1% of administered dose, however.

Several groups have suggested compartmental models to explain the uptake and metabolism of carnitine administered intravenously in dogs and rats (75,76,79). Brooks and McIntosh (79) proposed a three compartment model. The first tissue group was comprised of the liver, spleen, adrenal, and prostate. This group of tissues was thought to contain a compartment which exchanged carnitine rapidly with the plasma and a slowly exchanging compartment. In this group of tissues the rapidly exchanging compartment was thought to dominate. The second group of tissues, pancreas, seminal vesicle and testis contained fast and slow exchange compartments of equal size. Finally, the lung, skeletal muscle, and brain comprised a tissue group in which the slowly exchanging compartment dominated. The patterns of accumulation of radioactivity in the neonatal piglet tissues differ somewhat from the patterns observed by Brooks and McIntosh (79). In the present study, the spleen and adrenals had low levels of endogenous carnitine and negligible accumulation of radioactivity. The finding of low carnitine levels in adrenals has been confirmed in rats (79). Pancreas, testis, and seminal vesicles were not sampled in the present study. The presence and uptake of carnitine in the adult male reproductive tract is well established (80), however, the presence of carnitine in these organs of human neonates and neonatal piglets has not been established. The accumulation of radioactivity in the lungs and brain of the piglets was negligible.

Rebouche and Engel (76) proposed a three compartmental model comprised of plasma or extracellular fluid, skeletal and cardiac muscle, and liver and kidney. Like Brooks and McIntosh (79), these researchers based their model on the assumption that plasma comprised the circulating carnitine pool, providing carnitine to the tissues. The cardiac and skeletal muscle compartment was thought to be a slow-turnover pool that primarily accumulated carnitine. In contrast, liver and kidney made up a rapid-turnover pool, exchanging carnitine both into and out of the plasma. The patterns of radioactivity in the heart, muscle, liver and kidney expressed as dpm per mg noncollagen protein are displayed in Figures 5-11 to 5-14. In both the preliminary and final group of piglets, there is an increase in radioactivity from the intragastric dose in the heart and muscle tissue following tracer administration. Liver and kidney patterns also show an increase in tracer, but the patterns are more variable (Figures 5-11 and 5-12). The patterns of radioactivity accumulation from the intravenous dose in the heart and skeletal muscle show an accumulation similar to that observed with the intragastric dose. The liver and kidney patterns from the intravenous dose demonstrate a gradual decrease in the amount of radioactivity over time (Figure 5-13 and 5-14). This could be due to a more rapid

Figure 5-11: Radioactivity versus time from intragastrically administered radiolabeled carnitine in heart, muscle, liver, and kidney of the preliminary piglets.

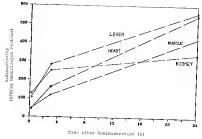

Radioactivity
(DPM/mg Noncollagen Protein)
600
500
400
300
200
100
0
0
4
8
12
16
20
24
Time after Administration (h)
LIVER
HEART
MUSCLE
KIDNEY

Figure 5-12: Radioactivity versus time from intragastrically administered radiolabeled carnitine in heart, muscle, liver, and kidney in the final group of piglets.

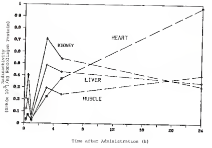

Radioactivity
(DPM(x 10³)/mg Noncollagen Protein)
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
HEART
KIDNEY
LIVER
MUSCLE
0
4
8
12
16
20
24
Time after Administration (h)

Figure 5-13: Radioactivity versus time from intravenously administered radiolabeled carnitine in heart, muscle, liver, and kidney of the preliminary piglets.

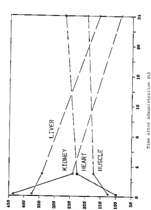
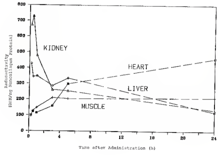

Radioactivity
(DPM/mg Noncollagen Protein)
450
400
350
300
250
200
150
100
50
LIVER
KIDNEY
HEART
MUSCLE
0
4
8
12
16
20
24
Time after Administration (h)

Figure 5-14: Radioactivity versus time from intravenously administered radiolabeled carnitine in heart, muscle, liver, and kidney of the final group of piglets.


Radioactivity
(DPM/mg Noncollagen Protein)
0
100
200
300
400
500
600
700
800
KIDNEY
HEART
LIVER
MUSCLE
0
4
8
12
16
20
24
Time after Administration (h)

appearance and clearing of radiolabeled carnitine in the plasma resulting from the intravenous administration.

In order to observe the relationship of the tissue accumulation of label to the amount of label in the plasma, the relative radiospecific activity (tissue radiospecific activity / plasma radiospecific activity) was calculated. These patterns are shown in Figures 5-15 to 5-18. The patterns observed in the liver and kidney, and heart and skeletal muscle are similar in both the preliminary and final groups of piglets. The patterns from both routes of administration are similar. Muscle and heart show a slower, more gradual accumulation while liver and kidney accumulation increases more rapidly.

The confirmation of any compartmental model for carnitine uptake and metabolism in the neonate will require further investigation. In addition, the question of whether carnitine is further metabolized or degraded to another metabolite in the neonate remains to be answered. The neonatal piglet is different from the animal models used in carnitine compartmental studies in terms of metabolic maturity. This is likely to be significant as evidence suggests a developmental aspect to both carnitine biosynthetic capacity (8) and to the enzymes of carnitine metabolism (51). Despite these considerations, the patterns of radioactivity resulting from radiolabeled tracer carnitine administration in the piglet tissues are similar to those

Figure 5-15: Relative radiospecific activity (tissue radiospecific activity/plasma radiospecific activity) versus time from intragastrically administered radiolabeled carnitine in heart, muscle, liver, and kidney of the preliminary piglets.

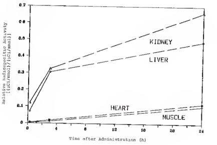

Relative Radiospecific Activity
[(µCi/mmol)/(µCi/mmol)]
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
KIDNEY
LIVER
HEART
MUSCLE
0
4
8
12
16
20
24
Time after Administration (h)

Figure 5-16: Relative radiospecific activity (tissue radiospecific activity/plasma radiospecific activity) versus time from intragastrically administered radiolabeled carnitine in heart, muscle, liver, and kidney in the final group of piglets.

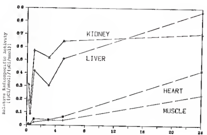

Relative Radiospecific Activity
[(µCi/mmol)/(µCi/mmol)]
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
KIDNEY
LIVER
HEART
MUSCLE
0
4
8
12
16
20
24
Time after Administration (h)

Figure 5-17: Relative radiospecific activity (tissue radiospecific activity/plasma radiospecific activity) versus time from intravenously administered radiolabeled carnitine in heart, muscle, liver, and kidney in the preliminary piglets.

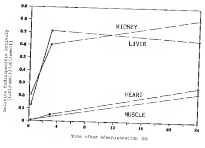
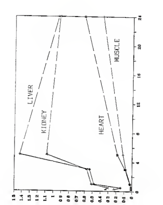

KIDNEY
LIVER
HEART
MUSCLE
Relative Radiospecific Activity
[(µCi/mmol)/(µCi/mmol)]
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
4
8
12
16
20
24
Time after Administration (h)

Figure 5-18: Relative radiospecific activity (tissue radiospecific activity/plasma radiospecific activity) versus time from intravenously administered radiolabeled carnitine in heart, muscle, liver, and kidney in the final group of piglets.


LIVER
KIDNEY
HEART
MUSCLE
1.5
1.4
1.3
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
4
8
12
16
20
24

observed in other species (76,80). Further investigation of carnitine compartmentation can now be accomplished using the neonatal colostrum-deprived piglet model for neonatal carnitine metabolism.

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

Conclusions

The primary goal of this project was the establishment of the neonatal colostrum-deprived piglet model for use in investigating neonatal nutrition and carnitine metabolism. A first step in this endeavor was to establish that the developmental patterns of tissue and blood total carnitine accretion in the fetal piglet are similar to those of the human fetus. The patterns of increasing levels of total carnitine with increasing gestational age in the skeletal muscle and decreasing levels towards fullterm gestation in the blood of the fetal piglet do resemble those patterns occurring in the human fetus. It is concluded that these similarities lend further support for the use of the neonatal piglet as an animal model for human neonatal carnitine metabolism.

A second step towards development of this animal model was the attempt to maintain colostrum-deprived piglets in a neonatal intensive care unit environment. This involved maintenance at a temperature range of 34 to 37°C, an environmental situation in which the metabolic energy demands for thermogenesis in the piglets should be low (10). This environment was similar to that of the human neonate in a neonatal intensive care unit. Clean room procedures, again similar to those observed in the neonatal intensive care unit, were followed. Feeding frequency of the piglet formula was modeled after the sow's normal feeding pattern. Under these conditions, the colostrum-deprived piglets maintained their body weights and were viable. Thus a second main conclusion of this project is that it is possible to maintain viable colostrum-deprived piglets without the use of germ-free or strict sterile techniques.

With the development of procedures allowing for successful maintenance of colostrum-deprived piglets, a final step in this project was the development and implementation of techniques for use in investigating neonatal nutrition and carnitine metabolism. Surgical catheterization techniques were developed which allowed convenient venous access for intravenous dosing or blood collection, intragastric access for controlled enteral dosing or sampling, and complete collection of urine. These techniques can be applied to a wide variety of neonatal nutritional investigations.

The techniques developed for use in the neonatal colostrum-deprived piglet have been used in an initial investigation of the tissue patterns of accumulation of radioactivity resulting from intravenous or intragastric administration of radiolabeled carnitine. It is concluded that the patterns of the appearance of radioactivity in the neonatal tissues, particularly the heart and skeletal muscle, and liver and kidney, are similar to patterns that have been observed in other species (76).

Recommendations

The techniques for the successful maintenance and use of this neonatal colostrum-deprived model developed in this project are now available. The piglet model more closely resembles the human neonate in physiology and metabolism than other non-primate animal models (9,10). Further use of this animal model to investigate neonatal nutrition and metabolism is recommended. Nutritional support techniques for use in human neonates, such as total parenteral nutrition, may be investigated and improved upon using this model. In addition, the metabolism and potential benefit to the neonate of certain nutrients, such as carnitine, taurine, or medium chain triglycerides can now be determined using the piglet model. Carnitine compartmentation in the neonate, and the influence of maturity and exogenous carnitine intake on these processes can now be examined. The influence of metabolic stresses, such as hypothermia or infection, on neonatal metabolism can be investigated in depth using this model. The piglet model can be used in whole animal in vivo studies, and can provide sample tissue for in vitro experimentation. Clearly, the establishment of an animal model useful for further study of the nutrition and metabolism of the neonate is the primary contribution of this project.

APPENDIX A

DETAILED ASSAY PROCEDURES

Carnitine Assay

Principle

This procedure is based on a modification of the procedure of Cederblad and Lindstedt (103). Using this assay, the recovery of carnitine added to plasma was 97 ± 12.5% while the recovery of carnitine added to muscle perchloric extracts was 103 ± 5.9% (103). The mean recovery of carnitine added to red blood cells determined with this assay was 98% (113), a plasma recovery value was determined to be 96% (113). Acetylcarnitine and acylcarnitine are hydrolyzed by alkaline hydrolysis. The total carnitine is measured by incubation of the sample with ^{14}C-acetylCoA and carnitine acetyltransferase. Samples containing radioactive tracer carnitine are assayed both with and without the addition of carnitine acetyltransferase. By subtracting the value obtained without addition of the enzyme from the value obtained with the enzyme it is possible to eliminate any counts resulting from carry over of the labeled carnitine. The radioactive acetylcarnitine is separated from the unreacted radioactive acetylCoA using a Dowex 2 x 8 anion exchange column. The radioactive acetylcarnitine fraction is quantitated by counting in a liquid scintillation counter.

Samples

Determinations are made on the following samples:

1. Plasma and erythrocytes: Blood is collected into an EDTA prepared tube. The hematocrit of the whole blood sample is first determined. Packed erythrocytes are separated from plasma by centrifugation (3,000 x g, 15 minutes). The plasma is collected and the packed cells are then resuspended in normal saline and recentrifuged. The supernatant is discarded, the cells are again suspended in saline and centrifuged again. The supernatant is discarded and the packed cells and plasma are stored frozen.
2. Urine samples are stored frozen and centrifuged (3,000 x g, 15 minutes) to remove solids prior to assay.
3. Tissues are frozen immediately upon collection and homogenized as described previously.

Reagents

1. Potassium hydroxide (KOH) 1 N: Dissolve 56.11 g of KOH (Fisher Scientific Company, Catalog #P-250) in 700 ml of deionized water. Bring final volume to 1 l with deionized water.

Potassium hydroxide (KOH) 4 N: Dissolve 224.44 g of KOH in 700 ml of deionized water. Bring final volume to 1 l with deionized water.

2. Perchloric acid 0.6 M. Add 193.5 ml of 70% perchloric acid (Fisher Scientific Company, Catalog #P-229) to 3,556.5 ml of deionized water.

3. Potassium phosphate dibasic (K_2HPO_4) 1 M: Dissolve 174.18 g of potassium phosphate dibasic (Fisher Scientific Company, Catalog #P-288) in 700 ml of deionized water, bring final volume to 1 l with deionized water.

4. Potassium phosphate monobasic (KH_2PO_4) 1 M: Dissolve 136.09 g of potassium phosphate monobasic (Fisher Scientific Company, Catalog #P-285) in 700 ml of deionized water, bring final volume to 1 l with deionized water.

5. Potassium phosphate buffer, pH 7.0 1M: Mix 1 M potassium phosphate monobasic with 1 M potassium phosphate dibasic until pH is 7.0. Store at 0 to 5°C.

6. N-ethylmaleimide 24 mM: Dissolve 21 mg of N-ethylmaleimide (Sigma Chemical Company, Catalog #E3876) in 7 ml of 1 M potassium phosphate buffer, pH 7.0. Make fresh on the day of the assay and discard unused portion. The N-ethylmaleimide is added to the assay mixture to pull the assay reaction to near completion and in this way make the standard curve a straight line (113).

7. Phenol Red 0.1%: Dissolve 0.1 g of phenol red (Fisher Scientific Company, Catalog #P-74) in 100 ml of 100%

ethanol. Add one drop of the solution to 10 x 75 glass test tubes and allow to dry. Store at room temperature.

8. (1-^{14}C)-acetyl coenzyme A: Dissolve 50 µCi of ^{14}C-acetylCoA (Amersham Radiochemicals, Catalog #CFA.452, 50 mCi/mmol) in 150 ml of cold deionized water. Dispense 15 ml aliquots into plastic scintillation vials and freeze at -70°C.

9. Potassium bicarbonate ($KHCO_3$) 1 M: Dissolve 1 g of potassium bicarbonate (Fisher Scientific Company, Catalog #P-184) in 7 to 8 ml of deionized water and bring the final volume to 10 ml. Make fresh on the day of assay and discard unused portion.

10. Acetic anhydride 0.1 M: Add 0.05 ml of acetic anhydride (Fisher Scientific Company, Catalog #A-10) to 4.95 ml of cold deionized water, mix and use immediately.

11. AcetylCoenzyme A 0.1 M: Dissolve 40 mg of Coenzyme A lithium salt (P.L. Biochemicals, Catalog #6200) in 2.0 ml of cold deionized water and mix. Add 0.4 ml of 1 M potassium bicarbonate and mix. Add 0.8 ml of 0.1 M acetic anhydride and mix immediately. Bring the final volume to 320 ml with deionized water. Dispense 15 ml into plastic vials and freeze at -20°C.

12. Carnitine acetyltransferase (Boehringer Mannheim Biochemicals, Catalog #103-241): Dilute the $(NH_4)_2SO_4$ solution with deionized water to a concentration of 25 units/ml. Store at 2 to 5°C. The activity of each new batch

of enzyme is tested by using at least 4 sets of standard curves and running an assay with the new enzyme batch. Control values assayed with the new enzyme must be within the calculated tolerance limits.

13. Ingredient Mixture: Prepare fresh on the day of the assay. Mix 2 volumes of (1-^{14}C)-acetylCoenzyme A, 1 volume of 24 mM N-ethylmaleimide and 1 volume of 0.1 mM acetylCoenzyme A.

14. Dowex columns: Place a small pledget of glass wool in 5 3/4 inch Pasteur pipets to form support for the resin bed. Add deionized water to Dowex 2 x 8, 200 to 400 mesh, chloride form anion exchange resin (BioRad Laboratories, Catalog #745-2451) until a slurry is formed that can just be pipetted with a Pasteur pipet. The resin is used to fill the columns to a height of 35 mm. Store the slurried resin at 2 to 5°C.

15. ACS Aqueous Counting Scintillant (Amersham Corporation, Catalog #196290).

Standards

L-carnitine hydrochloride (500 nmol/ml): Dissolve 9.88 mg of L-carnitine hydrochloride (General Biochemicals, Catalog #3363F, Lot #45882) in 70-80 ml of deionized water and bring the final volume to 100 ml with deionized water. Dispense 1 ml aliquots into 12 x 75 mm test tubes and store frozen at -70°C.

Instrumentation

Radioactivity is determined using a Beckman LS-2800 Liquid Scintillation System.

Directions for Performance of the Test

1. Prepare a hemolysate of the red blood cells by adding 0.5 ml of deionized water to 0.5 ml of packed, washed cells. Refreeze and thaw again.

2. Plasma and urine are assayed after centrifugation (3,000 x g, 15 minutes).

3. Prepare tissue samples by homogenizing 1 g of tissue in 3 ml of deionized water.

4. Prepare a carnitine assay write-up indicating the samples, standards, and controls to be assayed and their assay tube numbers.

A. 12 x 75 mm glass test tubes are numbered consecutively.

B. Unknowns are run in triplicate.

C. One incubation batch contains 60 to 90 samples.

D. A standard curve is run at the beginning of each incubation batch.

5. Following the protocol, prepare the standard curves as follows:

A. Thaw a tube of frozen standard and dilute it 1.4 ml plus 5.6 ml of deionized water.

B. Aliquot the following amounts of diluted standard and deionized water into 12 x 75 mm glass test tubes.

Tube #:	Carnitine Standard (ml)	Deionized Water (ml)	Carnitine (nmol)
1-10 and	0	225	0
61-70 and	12.5	212.5	1.25
121-130 and	25.0	200.0	2.50
181-190 and	37.5	187.5	3.75
etc.	50.0	175.0	5.00
	62.5	162.5	6.25
	75.0	150.0	7.50
	100.0	125.0	10.00
	125.0	100.0	12.50
	150.0	75.0	15.00

5. Following the protocol, pipet the correct amount of each unknown or control into the appropriate tubes.

Determination:	Analysate:
plasma carnitine	100 µl of plasma
erythrocyte carnitine	200 µl of hemolysate
urine carnitine	200 µl of urine
tissue carnitine	200 µl of homogenate

6. Bring the volume of all tubes to 225 µl with deionized water.

7. Add 25 µl of 1 N KOH, mix by vortexing and incubate at 50°C for 30 minutes. Cool to room temperature.

8. Add 750 µl of 0.6 M perchloric acid, mix by vortexing, centrifuge at 2,000-3,000 x g for 10 minutes.

9. Pipet 500 µl of each supernatant into a corresponding phenol red tube. Neutralize with 4 N KOH by dropwise addition (stop adding KOH when tubes turn pink).

10. Store in cold room overnight (0-5°C).

11. Centrifuge the phenol red tubes at 2,000-3,000 x g for 10 minutes.

12. Pipet 100 µl of each supernatant into a corresponding 10 x 75 mm glass test tube.

13. Add 100 µl of ingredient mix to each tube.

14. Add 40 µl of carnitine acetyltransferase to each tube at 20 second intervals (using a stopwatch) and incubate each tube at 37°C for exactly 30 minutes.

15. At the end of exactly 30 minutes at 37°C, remove 200 µl of each reaction mixture at 30 second intervals (using a stopwatch) and place in individual drained Dowex columns which are resting in individual 20 ml plastic scintillation vials.

16 Wash each column with two 0.5 ml aliquots of deionized water.

17. Add 9 ml of ACS aqueous counting solution to each vial, mix by hand, and place in the liquid scintillation counter with vials 1 to 12 in a rack with User #1 card in place.

18. To express erythrocyte carnitine in terms of hemoglobin, determine the hemoglobin content of the hemolysate using the Hemoglobin Procedure.

19. To express tissue carnitine in terms of protein, determine the noncollagen protein content of the homogenate using the Protein Procedure.

20. To express urinary carnitine in terms of creatinine, determine the creatinine content of the urine by the Creatinine Procedure.

Calculations

1. Obtain the counts per minute (cpm) from the counter printout.

2. Average the cpm corresponding to each point of the carnitine standard curve.

3. Subtract the cpm of the blank (0 carnitine point of the standard curve) from the cpm of each point of the curve.

4. Plot a standard curve of nmol of carnitine versus counts per minute and calculate the slope of the line.

5. Subtract the cpm of the blank from the cpm of each unknown.

6. Divide the corrected cpm of each unknown by the slope to determine the nmol of carnitine per volume of material assayed.

7. Multiply by the appropriate factor to determine the nmol of carnitine per ml of plasma, erythrocyte hemolysate, urine or tissue homogentate.

plasma	100 µl of sample	multiply by 10
erythrocytes	200 µl of sample	multiply by 5
urine	200 µl of sample	multiply by 5
tissues	200 µl of sample	multiply by 5

8. Make the further calculations as needed:

A. Erythrocytes: To calculate the nmol of carnitine per mg of hemoglobin, divide nmol of carnitine per ml of hemolysate by the mg of hemoglobin per ml of hemolysate.

B. Urine. To calculate nmol of carnitine per mg of creatinine, divide the nmol of carnitine per ml of urine by the mg of creatinine per ml of urine.

C. Tissues: To calculate nmol of carnitine per g wet weight, divide the nmol of carnitine per ml of homogentate by the g of tissue per ml of homogenate. To calculate nmol of carnitine per mg noncollagen protein, divide the nmol of carnitine per ml of homogenate by the mg of noncollagen protein per ml of homogenate.

Controls

Abnormal and normal controls for carnitine are not available. Periodically a large volume (100-200 ml) of blood is collected and prepared according to the procedure for collection for carnitine analysis. Aliquots of plasma and washed erythrocytes are frozen at -70°C and one aliquot assayed with each assay of unknowns. When only 20 aliquots of a particular batch remain, a new volume of blood is collected and prepared and the old and new controls are both assayed with each group of unknowns until none of the old controls remain. After determination of at least 20 consecutive values for a control batch, the mean ± 2 standard deviations is calculated. These define the tolerance limits of the assay. The calculated limits for the assays done in this project are as follows:

Control:	n	Mean ± SD	Lower Limit	Upper Limit
plasma (nmol/ml)	50	40.6 ± 9.4	21.8	59.4
red blood cells (nmol/mg hemoglobin)	41	0.25 ± 0.1	0.05	0.45

Hemoglobin Assay

Reagents and Solutions

1. Cyanmethemoglobin Certified Standard from Boehringer Mannheim Biochemicals. Concentration equal to 80 mg/dl.

2. Drabkin's Reagent: Prepare this reagent, from Drabkin's Dry Mixture obtained from Fisher Chemical Company, as described on the package.

Procedure

1. Centrifuge blood sample (3,000 x g) for 15 minutes.

2. Remove the plasma and add to the packed red blood cells several volumes of normal saline. Mix well by

inversion. Centrifuge (3,000 x g), remove the saline layer and repeat.

3. Decant the red blood cells into a 12 x 75 mm polypropylene tube and freeze at -70°C until assay.

4. Thaw the cells and add approximately an equal volume of deionized water.

5. Mix well and freeze and thaw once more.

6. Prepare a 1:10 dilution of this hemolysate by placing 100 µl into a 10 x 75 mm polypropylene tube containing 900 µl of deionized water.

7. Mix vigorously by vortexing for 10 seconds. (At this point, the tubes can be frozen at -20°C if necessary and assayed on a later date.)

8. Pipet 50 µl of the 1:10 hemolysate (mixed thoroughly just prior to pipetting) into duplicate 12 x 75 mm tubes.

9. Set up the standard curve in triplicate in 12 x 75 mm test tubes according to the following protocol:

Standard (ml)	Drabkin's Reagent (ml)	Hemoglobin (ml)
0	1.50	0
0.25	1.25	0.2
0.50	1.00	0.4
0.75	0.75	0.6
1.00	0.50	0.8
1.25	0.25	1.0
1.50	0	1.2

10. Add 1.45 ml of Drabkin's Reagent to each sample tube.

11. Allow the tubes to stand at room temperature for 15 minutes. If red particles are visible in the tubes at the end of this time, mix again and let the tubes stand for an additional 15 minutes.

12. Place the tubes on the batch sampler rack of the Beckman DU-7 Spectrophotometer as follows: one empty tube for back flush, one tube containing 1.5 ml of Drabkin's Reagent, 3 tubes of the first sample triplicate, one tube of deionized water (wash tube). Follow these tubes with next sample triplicate, wash tube etcetera. Every eleventh tube should be a recalibration tube containing Drabkin's Reagent.

Press the "single λ" key on the spectrophotometer. Enter OD = 540 nm, Filltime = 5 seconds. Enter the number of tubes. Press "start" key to calibrate the instrument. When the screen flashes "press run," press the "run" key to begin sample readings.

13. Plot a standard curve of mg of hemoglobin versus OD at 540 nm. Perform the calculations described in the following chart, average all intrasamples which are within 10%. Report the results as mg of hemoglobin per ml of hemolysate (mg/ml).

Operation:	Result:
1. OD--blank	
2. Slope	mg/50 µl of 1:10 diluted hemolysate
3. x 20	mg/total 1:10 diluted hemolysate = mg/100 µl of hemolysate
4. x 10	mg/ml hemolysate

Noncollagen Protein Assay

Principle

An alkaline copper solution is added to the solution containing protein. A complex with an absorption maximum at 310 nm forms between the protein bonds and the copper. The intensity of the blue color of this complex is proportional to the amount of protein present. Protein is quantitated by reference to a standard curve treated identically to the unkowns (104).

Samples

Determinations are made on tissue homogenates prepared for carnitine analysis.

1. Prepare an aliquot of each homogenate to remove collagen.

A. Muscle, heart, lung, intestine, stomach, brain, spleen, adrenals: Add 500 µl of homogenate to a 10 x 75 mm test tube containing 1000 µl of deionized water and 500 µl of 0.2 N NaOH.

8. Liver, kidney: Add 250 µl of homogenate to a 10 x 75 mm test tube containing 1250 µl of deionized water and 500 µl of 0.2 N NaOH.

2. Allow the tubes to sit overnight at room temperature.

3. Centrifuge each tube at 3,000 x g for 15 minutes.

4. Remove the supernatant and place in a clean 10 x 75 mm test tube.

5. Centrifuge the supernatant as above.

6. Remove the supernatant, place in a 10 x 75 mm test tube, cover with Parafilm. Refrigerate until assay.

Reagents

1. NaOH (0.2 N): Dissolve 8 g of sodium hydroxide in approximately 800 ml of deionized water. Bring the total volume to 1 l with deionized water.

2. NaOH (40 %): Dissolve 1200 g of sodium hydroxide in deionized water. Bring the total volume to 3 l.

3. Microbiuret-copper solution: Add 800 ml of deionized water to a 40% NaOH solution as prepared above. Filter through #1 Whatman filter paper. Let the solution stand at least one week before use.

4. Copper sulfate (1%): Dissolve 8 g of $CuSO_4 \cdot 7H_2O$ in deionized water and bring the final volume to 800 ml with deionized water.

5. Microbiuret + copper solution: Add 800 ml of 1% copper sulfate (as prepared above) to a 40% NaOH solution as

prepared above. Add very slowly dropwise using a separatory funnel for the copper sulfate. Stir the NaOH continuously. Caution: the copper sulfate will precipitate out of solution if not added very slowly. Filter through #1 Whatman filter paper. Let the solution stand for at least one week before use.

Standards

Bovine serum albumin standard solution: Bring 250 µl of Bovine Albumin (10% solution obtained from Pentex, Miles Laboratories) up to 25 ml with deionized water. Mix very well, but very carefully. Use to determine a standard curve and discard. A standard curve may be determined once for each batch of microbiuret-copper and microbiuret + copper reagents.

Set up the standard curve in 10 x 75 mm test tubes as follows, in triplicate:

Standard (ml)	Water (ml)	Micro-Cu (ml)	BSA (µg)
0	400	800	0
25	375	800	25.5
50	350	800	51.0
75	325	800	76.5
100	300	800	102.0
125	275	800	127.5
150	250	800	153.0
175	225	800	178.5
200	200	800	204.0
225	175	800	229.5
250	150	800	255.0
275	125	800	280.5
300	100	800	306.0
325	75	800	331.5
350	50	800	357.0
375	25	800	382.5
400	0	800	408.0

Standard (ml)	Water (ml)	Micro+Cu (ml)	BSA (µg)
0	400	800	0
25	375	800	25.5
50	350	800	51.0
75	325	800	76.5
100	300	800	102.0
125	275	800	127.5
150	250	800	153.0
175	225	800	178.5
200	200	800	204.0
225	175	800	229.5
250	150	800	255.0
275	125	800	280.5
300	100	800	306.0
325	75	800	331.5
350	50	800	357.0
375	25	800	382.5
400	0	800	408.0

Mix all tubes very carefully by inversion. Let the tubes sit at room temperature for 30 minutes. Determine the absorbance at 310 nm on the Gilford 2400 spectrophotometer. Determine the slope of the standard curve as follows.

1. Average the absorbances of all triplicates.

2. Subtract the Micro-Cu blank absorbance from all Micro-Cu standard absorbances and the Micro+Cu blank absorbance from all Micro+Cu standard absorbances.

3. Subtract each Micro-Cu standard absorbance from its corresponding Micro-Cu absorbance. This is the corrected absorbance.

4. Plot a curve of µg BSA versus corrected absorbance at 310 nm.

5. Determine the slope of the line.

Instrumentation

Absorbances are determined on the Gilford 2400 spectrophotometer at 310 nm

Directions for Performance of the Test

1. Prepare a protein write-up sheet.

2. Prepare 4 Micro-Cu blanks by adding 400 µl of deionized water to 4 10 x 75 mm test tubes labeled as -Cu blanks.

3. Prepare 4 Micro+Cu blanks by adding 400 µl of deionized water to 4 10 x 75 mm test tubes labeled as +Cu blanks.

4. Prepare 4 Micro-Cu assay tubes for each sample by placing 300 µl of deionized water and 100 µl of supernatant in 10 x 75 mm test tubes.

5. Prepare 4 Micro+Cu assay tubes for each sample by placing 300 µl of deionized water and 100 µl of supernatant in 10 x 75 mm test tubes.

6. Add 800 µl of Micro-Cu solution to the appropriate blank and sample assay tubes.

7. Add 800 µl of Micro+Cu solution to the appropirate blank and sample assay tubes.

8. Mix gently.

9. Let all tubes stand for 30 minutes at room temperature.

10. If liver samples are assayed, let the tubes sit 30 minutes, than mix again and let sit another 30 minutes.

11. Determine the absorbance at 310 nm on the same instrument on which the standard curve was determined.

Calculations

1. Average the absorbances of all quadruplicates.

2. Subtract the Micro-Cu blank absorbance from the absorbances of all Micro-Cu unknowns.

3. Subtract the Micro+Cu blank absorbance from the absorbances of all Micro+Cu unknowns.

4. Subtract each Micro-Cu unknown absorbance from its corresponding Micro+Cu unknown absorbance. This is the corrected absorbance.

5. Continue the calculations as follows:

Operation	Result
A. Tissue	
1. Corrected absorbance x 1/slope	μg/100 μl of diluted homogenate
2. x 10	μg/ml of diluted homogenate
3. divided by 1000	mg/ml of diluted homogenate
4. x 4 (all tissues except liver and kidney) x 8 (liver and kidney)	mg/ml of homogenate

Creatinine Assay

Principle

The levels of creatinine in urine samples are measured using the Sigma Diagnostic Kit on the Beckman DU-7 spectrophotometer. Creatinine reacts with Alkaline Picrate Solution tp yield a yellow-orange color. The addition of an acid reagent destroys the color. The difference of color intensity measured at 500 nm before and after acidification is proportional to creatinine concentration.

Reagents

1. Creatinine Standard Solutions, Sigma Chemical Company.
2. Acid Reagent: Sulfuric and acetic acid, Sigma Diagnostic Kits.
3. Sodium Hydroxide (1.0 N)
4. Alkaline Picrate Solution, Sigma Diagnostic Kits.

Procedures

1. Put 2 ml of each urine sample in a 12 x 75 mm test tube and centrifuge for 10 minutes at 2,000 - 3,000 x g.
2. Make a 1:10 dilution of each urine sample, using 100 µl of urine and 900 µl of deionized water.
3. Make the Alkaline Picrate Solution by mixing 5 volumes of Creatinine Color Reagent with 1 volume of Sodium Hydroxide Solution.

4. Put 0.3 ml of deionized water in Blank tubes (13 x 100 mm test tubes labeled 1,2,3). Put 0.3 ml of the Creatinine in Standard Solutions (3,5,7 and 10 mg/dl in dilute acid) in triplicate into tubes 4 to 15.

5. Add 0.3 of diluted sample in triplicate to sample tubes.

6. Add 3 ml of the Alkaline Picrate Solution to all tubes. Mix and allow to stand at room temperature for 8 to 12 minutes.

7. Place tubes on batch sampler rack of the spectrophotometer as follows: one empty tube (for back flush), one tube containing 3 ml of deionized water (Blank) followed by standard and the sample triplicates. Place a tube filled with water after every eleventh tube. Press the "single λ" key on the screen of the spectrophotometer. Enter in Absorbence = 500 nm, Filltime = 5 seconds. Enter number of tubes in assay.

8. Press "Start" key to calibrate the instrument. When screen flashes "Press Run," press the "Run" key to begin sample readings.

9. After recording initial absorbance (IA), add 0.1 ml of the Acid Reagent to all tubes. Mix and allow to stand at room temperature for 5 minutes.

10. Repeat step #8 to read and record the final absorbance (FA).

Analysis

1. Find the average IA for each standard and sample.

2. Find the average FA for each standard and sample.

3. Subtract the average FA from the average IA for each standard and sample.

4. From the standard values, calculate the correlation coefficient, slope of the line, and Y intercept. Graph the standard curve.

5. Divide the OD of each sample by the calculated slope. This gives mg creatinine/dl.

6. Adjust each value by multiplying by 10 to account for the 1:10 dilution.

7. Divide each value by 100 to obtain the value as mg creatinine/ml. This is the final value. Record in Creatinine Book and retain the write-up.

8. Tape printouts to assay write-up, place in Creatinine Write-up Notebook.

APPENDIX B

COMPOSITION OF PIGLET DIET FORMULA

Table B-1
Composition of Piglet Formula and Sow's Milk

Nutrient	Double-Strength Formula	Sow's Milk (111)
Protein	40 g/l	72 g/l
Fat	72 g/l	61 g/l
Carbohydrate	136 g/l	48 g/l
Ash	8 g/l	9.6 g/l
Folic Acid	213.4 µg/l	3900 µg/l
Thiamin	1056 µg/l	650 µg/l
Riboflavin	1268 µg/l	1370-3200 µg/l
Niacin	17 mg/l	4.3-9 mg/l
B6	845.6 µg/l	200 µg/l
B12	4.2 µg/l	1.4 µg/l
Biotin	105.8 µg/l	14 µg/l
Pantothenic Acid	6.34 mg/l	4 mg/l
Vitamin A	1268 µg/l	150-2550 µg/l
Vitamin D	21.2 µg/l	2.5 µg/l
Vitamin E	42 mg/l	1 mg/l
Vitamin C	110 mg/l	146 mg/l
Calcium	1268 mg/l	2100 mg/l
Phosphorus	1004 mg/l	1000-1900 mg/l
Potassium	1564 mg/l	1000 mg/l
Sodium	581.4 mg/l	340 mg/l
Magnesium	148 mg/l	200 mg/l
Chloride	1100 mg/l	1000 mg/l
Iron	25.4 mg/l	1.33 mg/l
Zinc	10.6 mg/l	4.94 mg/l
Manganese	0.422 mg/l	-
Copper	1.27 mg/l	-
Iodine	0.138 mg/l	-
Kilocalories	1.35 Kcal/ml	1.03 Kcal/ml

Note: Piglet Formula #1 is double-strength Prosobee (Mead Johnson Nutritional Division, Evansville, IN) 122 g in 1 l of sterile deionized water.

Table B-2
Composition of Piglet Formula #2

Ingredient	Quantity for One Liter of Formula
Prosobee	122 g
Casein	25 g
AIN-76 Mineral Mix	3 g
Corn Oil	35 ml

Note: Ingredients are blended with sterile water and volume is brought to one liter. Casein is assayed carnitine-free.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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